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Volume 53 AUGUST, 1953 Number 2

ENDOCRINOLOGY

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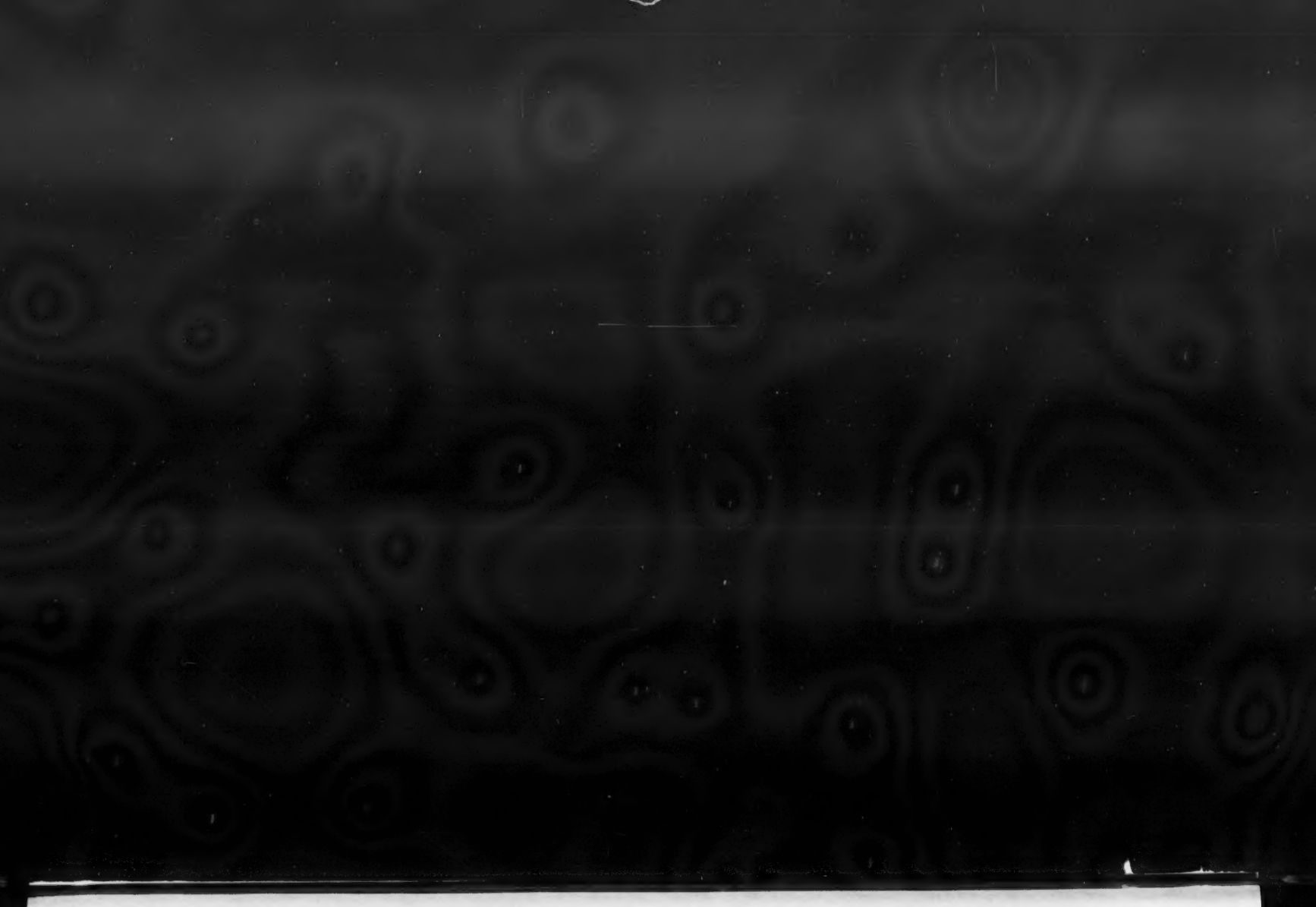

ENDOCRINOLOGY

VOLUME 53 AUGUST, 1953 NUMBER 2

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ELECTRON MICROSCOPIC OBSERVATIONS ON THE THYROID

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THIS paper reports some observations on sections of rat and guinea pig thyroids in various states of secretory activity.

MATERIALS AND METHODS

In all experiments guinea pigs of 180–220 gm. weight and Wistar strain rats of 140–160 gm. weight were used. Preparations of thyroid stimulating hormone (TSH) and thyroxine, supplied through the courtesy of Hoffman-La Roche A. G. Basel, Switzerland, were injected subcutaneously. Thiouracil pellets were implanted under the skin. Hypophysectomy was performed by the parapharyngeal approach.

The thyroids were removed under ether anesthesia and immediately fixed for 24 hours in a 2% solution of OsO_4 in buffered Tyrode at a pH of 7.5. Thereafter the pieces were rinsed for 1 hour in flowing tap water and transferred for dehydration successively into 50, 70, 96 and two changes of 100 percent ethanol, being kept for 1 hour in each. After remaining in benzol for 2 hours, the pieces were embedded in "esterwax" at 52° C. for 24 hours. Then they were kept at 5° C. for 20 hours and at −20° C. for 5 hours.

A Cambridge rocking microtome was used. The sectioning was performed at 5° C. A smooth advance for very fine sectioning was obtained by the slight extension of the metallic block according to the change of temperature from −20° C. to 5° C. The sections from the knife were floated on water to facilitate spreading. From there they were lifted on electron microscope screens of fine copper mesh coated with a film made from a 0.1% "Formvar" solution. Then they were placed on filter paper, gently immersed in xylol in order to remove the "esterwax" and allowed to dry.

The sectioned material was examined in a Philips (model 1951) electron microscope. The micrographs were taken at an original magnification of 5000× or 10,000×. The accelerating voltage was generally 60 KV.

OBSERVATIONS

Figures 1 and 2 show a normal follicle of guinea pig thyroid. The well-known histologic structures can be recognized. The mitochondria and

Received for publication February 14, 1953.

osmiophilic granules in the cytoplasm and the terminal bars show distinctly. There is no material which would correspond to the Golgi apparatus. Two previously unknown structures are to be observed:

1) The free surface of the follicular cells shows very fine filamentous projections, about 0.4μ long and 0.05–0.08μ wide.

2) In the cytoplasm of some of the follicular cells there are characteristic canalicular elements. These lie generally parallel and are 0.05–0.08μ wide and up to several microns long. A considerable number of these may be either concentrated around the nucleus or form whorl-like figures (Fig. 3).

In the rat thyroid the canalicular cytoplasmic structure is much more abundant than in the guinea pig. Frequently it penetrates the whole cytoplasm, as a mesh.

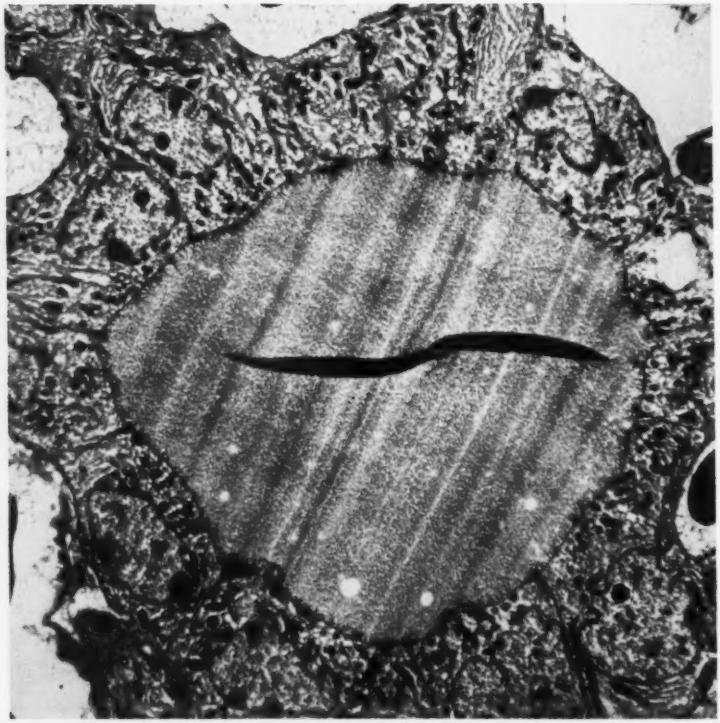


FIG. 1. Follicle of normal guinea pig thyroid. A moderate amount of canalicular structure is seen in some cells. Fine protrusions are seen on the free surface of the cells. The nuclei, nucleoli, mitochondria, osmiophilic droplets, and terminal bars are clear. No Golgi apparatus is seen. ×3000.

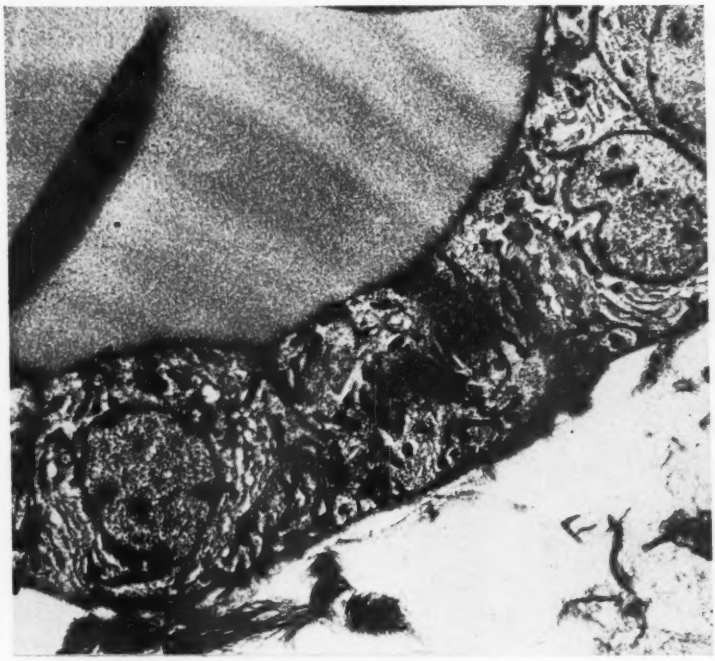


FIG. 2. Part of a follicle of normal guinea pig thyroid. ×5000.

THE INFLUENCE OF TSH

Figures 4–9 show the thyroids of guinea pigs 1, 6, 12, and 24 hours after injection of 10 I.U. of TSH. First (Figs. 4 and 5) the cells show cytoplasmic droplets and blebs at the free surface. The canalicular structure in the cytoplasm is inconspicuous and the number of the fine projections on the free cell surface appears reduced. After 6 hours columnar cells with numerous projections are seen (Figs. 5 and 6). The cytoplasm remains clear. The canalicular structure is again present. After 12 hours the fine projections are again clearly discernible (Figs. 7, 8 and 9). The cytoplasm has a washed-out appearance. The blood vessels appear enlarged.

With higher doses of TSH (e.g. 25 units) there may occur a breakdown of follicular cells, and blood appears in the follicle (Fig. 10).

Similar morphologic changes of the follicular cells are seen in rat thyroid. However, the stages are not as distinctly separated, and the loss of the canalicular structure in the cytoplasm shortly after the injection is not as pronounced (Fig. 11).

FIG. 3. Follicular cells of normal guinea pig thyroid showing marked canalicular structure in the cytoplasm. ×8000.

THE INFLUENCE OF HYPOPHYSECTOMY

Following hypophysectomy the follicular cells become flat, the canalicular cytoplasmic structure and the intrafollicular projections diminish.

The same morphologic changes can be observed after treatment with thyroxine.

THE INFLUENCE OF THIOURACIL

Ten days after subcutaneous implantation of 25 mg. of thiouracil the height of the follicular cells increases, the canalicular cytoplasmic structure appears more marked.

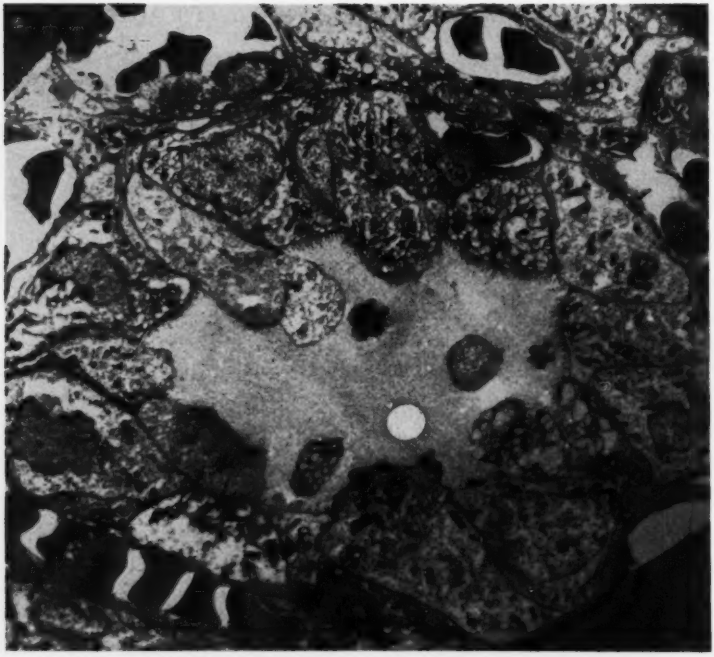
DISCUSSION

In connection with the fine filamentous projections from the free surface of the follicular cells, a similar submicroscopic structure has been seen in epithelial cells of the gall bladder, in the thin limb of Henle's loop, and in

FIG. 4. Part of a follicle of guinea pig thyroid 1 hour after the injection of 10 units of TSH. The cytoplasm is filled with droplets, some extending into the follicular lumen. No canalicular structure is seen. ×3500.

small bile ducts. (Dalton *et al.*, 1951; Braunsteiner *et al.*, 1953b). It thus seems likely that such structure may be related to the absorption of fluids.

The canalicular cytoplasmic structures described here are evidently identical with those previously described by Dalton *et al.* (1950, 1951), Bernhard *et al.* (1952), and Braunsteiner *et al.* (1953a) in liver cells, pancreatic exocrine cells, cells of the salivary gland, pepsin producing cells of the stomach and plasma cells of the bone marrow. Apparently the presence of this structure in these cells is associated with a high content of ribonucleoproteins (RNP). Bernhard *et al.* (1952) believe these canaliculi to represent RNP, that is, the ergastoplasm of the classics. We are not entirely convinced of this, as we have seen other cells rich in RNP, for instance tumor cells and immature blood cells, in which such canaliculi could not be demonstrated. These structures seem to be closely related to protein



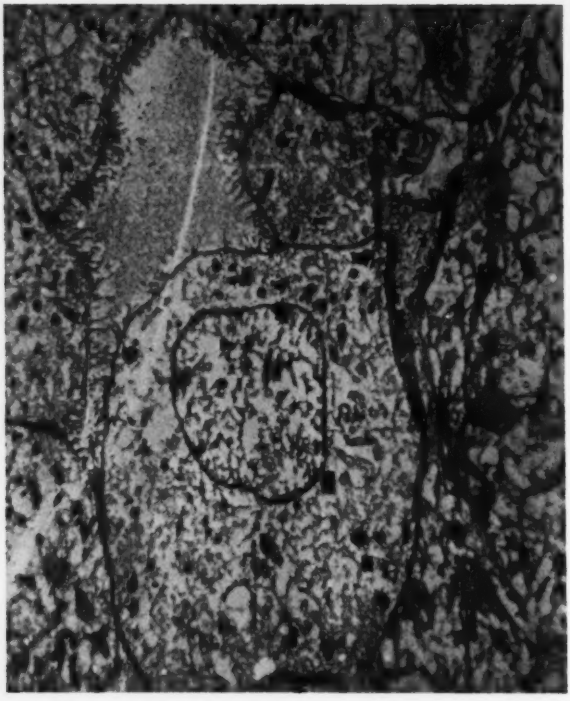


FIG. 5. Follicle of guinea pig thyroid 6 hours after the injection of TSH. Some cells show traces of canalicular structure in the cytoplasm. Remnants of follicular cells are floating in the colloid. The capillaries are enlarged. ×3000.

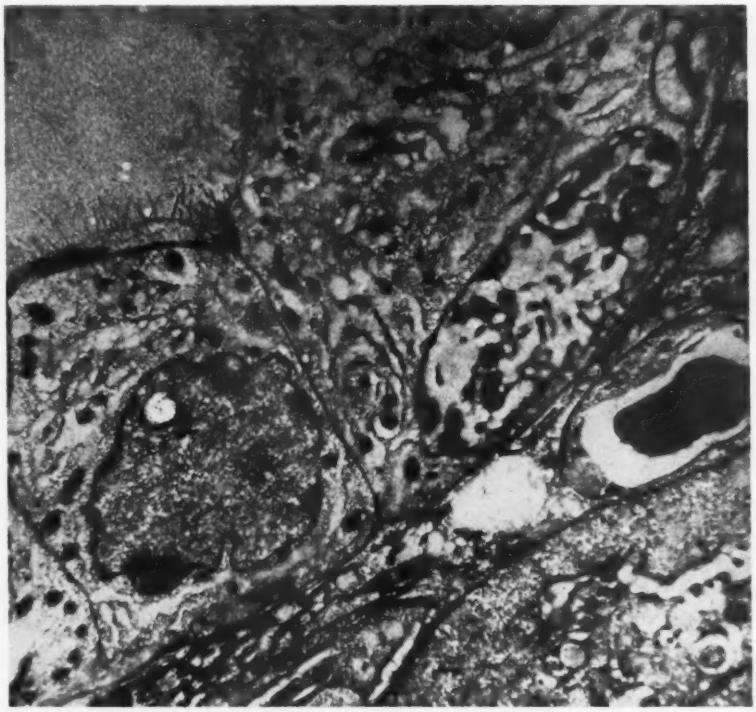
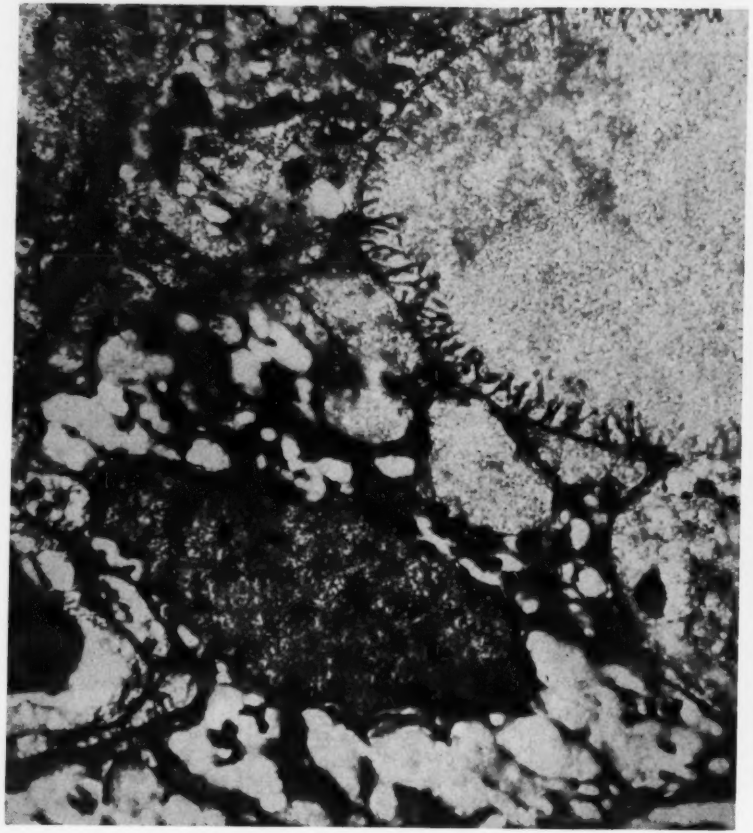


FIG. 7. Follicular cells of guinea pig thyroid 12 hours after the injection of TSH. The cells appear rather empty. ×8000.

synthesis and excretion, because cells now known to have this structure all excrete specific proteins (Braunsteiner *et al.*, 1953 a).

The description of the response to TSH suggests a distinct secretion of colloid into the follicular lumen during the first hours. The accompanying loss of filamentous cytoplasmic structure is similar to that observed by

FIG. 6. Follicle of guinea pig thyroid 8 hours after the injection of TSH. The follicular cells are columnar, canalicular structure in the cytoplasm is not marked, but the intrafollicular projections are augmented. ×5000.



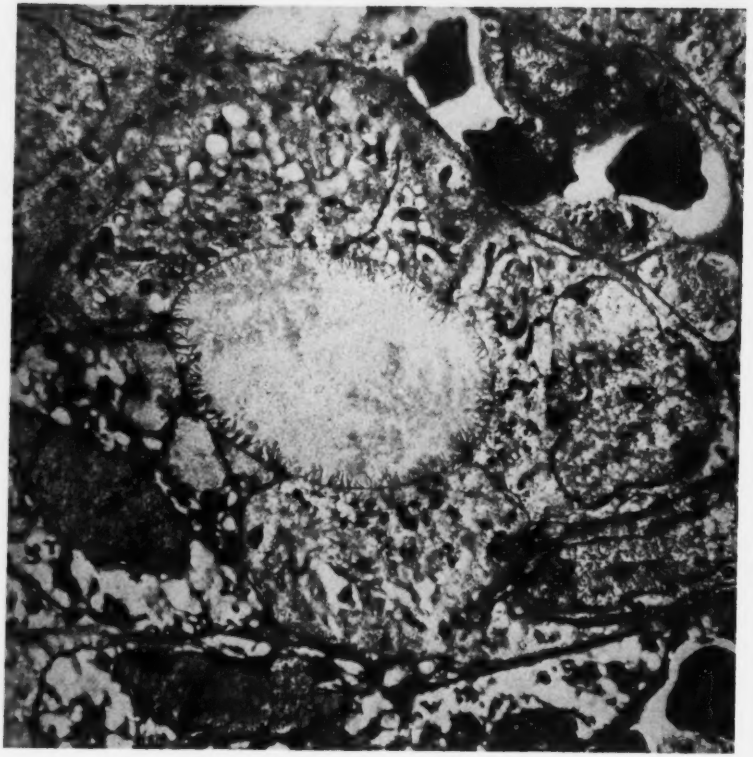


FIG. 8. Follicle of guinea pig thyroid 12 hours after the injection of TSH. The intrafollicular projections are very distinct. ×4000.

FIG. 10. Degenerated guinea pig follicular cells 24 hours after the injection of 25 units TSH. Erythrocytes are seen in the follicular lumen. ×7000.

Gautier (1953) in cells of the salivary gland after the injection of pilocarpine. In accord with the observations of Leblond and Gross (1948) obtained with radioiodine we have never found changes which could be interpreted as a reversal of polarity in the cell with a direct secretion into the blood stream (Bensley, 1916; and others). The empty appearance of the cytoplasm and the augmented projections of the free surface of the follicular

FIG. 9. Part of fig. 8. The cells have a washed-out appearance. ×8000.

cells that are evident a few hours later may be a result of the reabsorption of liquefied colloid and its release into the circulation.

The observations with thiouracil resemble those seen following prolonged treatment with small doses of TSH.

After hypophysectomy the canalicular structure diminishes in the fat-

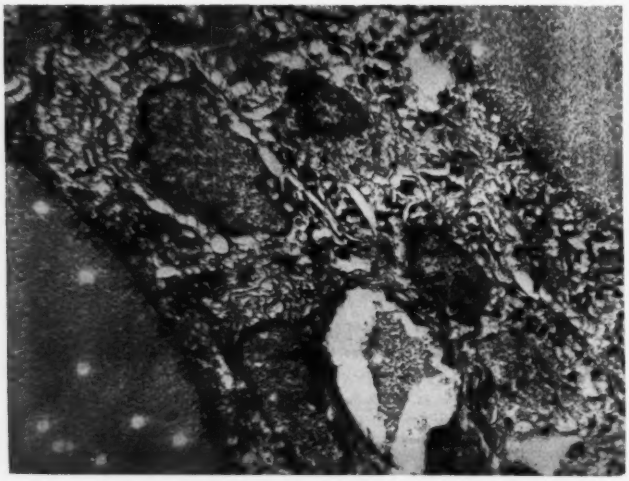


FIG. 11. Part of a follicle of rat thyroid 5 days after daily treatment with 10 units TSH. The canalicular structure of the cytoplasm is abundant, the filamentous projections well developed. ×4000.

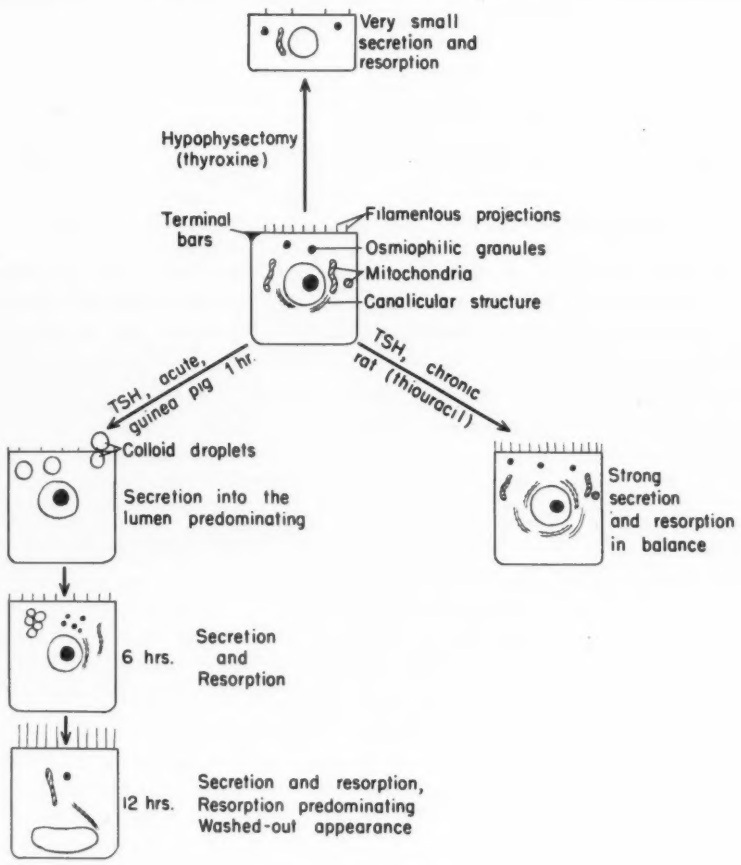
tened cells. This may be related to the diminished protein hormone production and excretion.

The accompanying diagram summarizes the above material.

SUMMARY

Sections of the thyroid of guinea pigs and rats were examined under the electron microscope. Thyroids were taken from normal and hypophysectomized animals and from animals treated with thyrotropic hormone, thyroxine or thiouracil.

In the follicular cells a submicroscopic canalicular structure of the cytoplasm could be demonstrated. The appearance of this structure seems related to the functional state of the gland. The cells show fine intrafollicular projections that seem augmented during reabsorption of liquefied colloid. No evidence of direct hormone secretion in the bloodstream was observed.



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EFFECTS OF GROWTH HORMONE IN DOGS[1]

JAMES CAMPBELL, H. R. HAUSLER, J. S. MUNROE AND I. W. F. DAVIDSON

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IT WAS found by Campbell, Lei and Davidson (1951) that the administration of purified bovine growth hormone to dogs caused an increase of the blood erythrocyte sedimentation rate (ESR), which was much greater than could be accounted for by the coincident reduction in the volume per cent of packed red blood corpuscles. Evidence was obtained that the fibrinogen content of the blood plasma was also increased by the growth hormone. These effects coincided with, and followed fairly closely, the considerable hyperglycaemia and glycosuria produced by the injections. These observations have been confirmed, and changes produced in the blood and tissues of dogs by doses of growth hormone adequate to produce idiohypophyseal diabetes have been studied.

The principal findings are, in brief, that in the dogs given growth hormone, the quantity of protein in the blood plasma is increased, and the plasma protein pattern is altered. The amount of fibrinogen is increased due to increased concentration and increase in plasma volume. The amount of other globulins is increased, due to some increase in concentration and the increase in plasma volume. The quantity of red cells in the blood is not decreased, but the volume per cent of packed cells is decreased due to dilution by the increased volume of plasma. The activity of the clotting system is increased since the clotting time of the blood, and the prothrombin time of the plasma decreased. A leucocytosis occurred, due to increases in neutrophil granulocytes and in stab cells.

METHODS

Venous blood was collected in cooled siliconed syringes and was transferred quickly to test tubes in an ice-bath. The first sample of 1 ml. was collected in a syringe containing 2 mg. of finely powdered sodium oxalate. The needle was left in place and two constricted capillary tubes were filled with blood directly from the needle for the determination of the clotting time by the method of Hausler (1953). The next sample, which was usually 2 ml. (but 4 ml. when blood volume was determined) was collected in a syringe containing 1 drop of heparin solution (1000 units per ml.) per 2 ml. blood. The plasma volume was determined by the dye method (Gregerson, 1944). A filtered, sterile 0.25% solution

Received for publication February 20, 1953.

[1] This investigation was aided by a grant from the National Research Council of Canada, and from the National Cancer Institute of Canada.

of Evans blue dye (T 1824) in pyrogen-free saline was sealed in 3.5 ml. ampoules, enough stock solution being prepared to last for the duration of the experiment. After withdrawing the initial sample of blood, 2.0 ml. of the dye solution was injected intravenously by a calibrated syringe. After 10 minutes another sample of blood was withdrawn into a syringe containing heparin. The dye in the plasma of the blood sample was determined photometrically at 600 mμ, plasma from the initial sample serving as a blank. A reference standard dilution curve of the stock dye solution was prepared, readings being made with a Coleman model 6A Spectrophotometer, using micro-cuvettes.

The ESR of heparinized blood was determined at 25° C. in Wintrobe tubes which were capped and suspended vertically in water contained in a flat-sided glass specimen jar. Readings were made at 10-minute intervals for 1 hour. The haematocrit readings were taken after centrifuging for 30 minutes at 3000 r.p.m., the radius from the axis of the centrifuge to the 5 cm. mark on the tubes being 17 cm. Oxyhaemoglobin was determined by the optical density at 545 mμ of 0.02 ml. blood in 6 ml. of 0.1% Na_2CO_3 solution, measured in small cuvettes (operating directions, Coleman model 6A). The densities of whole blood and plasma were determined by the copper sulfate method of Phillips *et al.*, (1950). The viscosities of whole blood and plasma were determined with a Hellige Viscometer.[2]

The methods described by Fowler (1945) were used in making red and white cell counts. In the determination of reticulocytes (Magmer, 1938), a white blood cell diluting pipette was filled to mark 0.3 with a 1% solution of brilliant cresyl blue in saline, and blood was drawn in until the fluid came to mark 0.6. The fluid was transferred to a porcelain plate, mixed, and after 20 seconds was smeared on slides. The dried smears were counter-stained with Wright's or Leishman's stains.

The nitrogenous fractions of the plasma were determined by minor modifications of the methods described by King (1951). The plasma total protein nitrogen was determined as the difference between the total and the non-protein nitrogen. The albumin nitrogen was determined after precipitation of the globulins by 21% Na_2SO_3. Fibrin nitrogen was determined in a separate sample of plasma. The globulins were calculated by difference as total protein minus the sum of albumin plus fibrin, i.e., the total protein of the plasma equals albumin plus globulin plus fibrin. The details are, non-protein nitrogen: to 0.2 ml. heparinized plasma in 3.20 ml. distilled water, added 0.6 ml. of 25% trichloracetic acid. The nitrogen was determined in 1 ml. aliquots of the filtrate. Total nitrogen:—to 0.2 ml. oxalated plasma added 9.8 ml. of 0.9% NaCl and determined the nitrogen in 0.2 ml. of the solution. Albumin (modification of the method of Campbell and Hanna, 1937): to 0.2 ml. plasma added 9.8 ml. of 21% Na_2SO_3 (Merck reagent, anhydrous) and mixed. After standing 9 minutes added 1 drop of caprylic alcohol, mixed and filtered after 2 minutes through Whatman #42 paper, refiltering the first 2 ml. of filtrate. To 0.5 ml. of the clear filtrate added 0.6 ml. of 25% trichloracetic acid and 1 ml. distilled water. Centrifuged, decanted the clear supernatant, drained by inversion and determined the nitrogen of the precipitate. Fibrin: to 0.4 ml. of oxalated plasma added 19.6 ml. of the borate buffer described by Ferguson (1943) which is composed of 45 parts of 2.5% boric acid solution, 10 parts of 4% sodium tetraborate solution and 45 parts of 0.5% NaCl solution. The pH was adjusted to 7.4. To 4 ml. of the diluted plasma added 0.2 ml. of a "clotting" solution which contained per 100 ml.: "Clotting Globulin,"[3] Lederle, 2.5 gms., NaCl 1.0 gm., sodium acetate 0.7 gm., and calcium acetate 1.8 gm. After mixing

[2] Hellige Inc., Long Island City, N.Y.

[3] Donated by Dr. H. D. Piersma, Lederle Laboratories, Division of American Cyanamid Co.

and incubating for 10 minutes at 37° C. the fibrin strands of the clot were wound on a fine glass rod. The fibrin and the test-tube were washed with distilled water, the test-tube was drained and the portion of the rod with the adherent fibrin was returned to it for the determination of nitrogen. This solution was used in order to accelerate clot formation, since the rates of fibrinogenolysis and of fibrinolysis were also determined.

The rate of fibrinogenolysis was determined by incubating in test-tubes at 37° C. 4 ml. samples of the oxalated plasma which had been diluted 2/100, v/v (as described above) with borate buffer at pH 7.4. At 1, 3 and 6 hours, 0.2 ml. of the clotting solution was added, mixed and after 10 minutes further incubation the fibrin was collected for determination of nitrogen as described above. For the determination of the rate of fibrinolysis the clotting solution was added to all the plasma-borate buffer samples prior to incubation so as to produce the fibrin clot. After 1, 3, and 6 hours at 37° C. the remaining fibrin was removed and determined.

The samples for the determination of nitrogen were digested with 0.2 ml. of 50% H_2SO_4, a drop of H_2O_2 solution being added during this process. After cooling, 6.9 ml. of distilled water was added, followed by 3 ml. of the Nessler reagent according to Folin and Wu (1919). The optical density of the solutions was determined at 520 mμ in the Coleman model 6A Spectrophotometer. Solutions prepared from repeatedly recrystallized ammonium sulphate and from digestion of a casein (Hammarsten) preparation were used for the preparation of the standard curves.

The prothrombin time was determined according to Quick (1938), using 0.1 ml. samples of oxalated plasma. Sugar was determined in 0.1 ml. samples of blood by Nelson's chromogenic reagent method, according to Somogyi (1945). All these determinations could be made on 7 ml. of blood, and when blood volume was not done, on 4 ml. The insulin extractable from the pancreas was determined by the mouse-convulsion method (Wrenshall, 1951). The total lipid and fat-free solids of the tissues were determined according to Best, Lucas, Patterson and Ridout (1946).

The following methods were used in the analysis of urine. Total nitrogen by the micro-Kjeldahl method of Folin and Farmer (1912); urea by the method of Marshall described by Kolmer and Boerner (1945); uric acid according to Folin (1933, 1934); creatinine by the method of Folin (1904), described in detail by Bonsnes and Taussky (1945); sugar by Benedict's method (1911); and albumin by the sulfosalicylic acid method of King (1951).

The growth hormone preparation D21P was obtained from bovine anterior pituitary glands by the method of Campbell and Davidson (to be published). The growth-promoting activity was measured by the tibia test of Greenspan, Li, Simpson and Evans (1949) in young, female hypophysecotmized Sprague-Dawley rats. The environmental temperature was 29 $\pm$ 0.5° C. and the diet consisted of whole milk, whole wheat bread and ground lean meat in the proportions by weight of 2.5:1:2. Doses of 10 and 25 μg. per rat per day for 4 days increased the width of the uncalcified disc of the proximal epiphysis of the tibia over the control value by 116 and 149 microns respectively. This response compares favourably with that obtained by Greenspan *et al.* (1949) with bovine growth hormone, but the assay results cannot be compared directly as they were obtained in different laboratories. The thyroid-stimulating hormone (TSH) activity of D21P, assayed by the method of Ghosh, Woodbury and Sayers (1951),[4] was found to be 0.10 U.S.P. units per mg. The prolactin activity was estimated by the local cropsac response of Hall (1944), to be about 5% of that of the International Standard Prolactin, which

[4] Assay by the Salt Lake Laboratories, Inc., Salt Lake City, Utah.

was supplied by the U.S.P. The ACTH content, assayed by the method of Sayers, Sayers and Woodbury (1948) was of a low order.[5] The solutions for injection contained 10 mg. of the lyophil-dried growth hormone preparation per ml. in sterile, pyrogen-free saline about pH 8.

Regimen

The four intact, adult test dogs (F, P, M and R of body weight 11.2, 9.4, 9.8 and 12.2 kg. respectively) were accustomed to cage life, and during preliminary or control period of 30 days, 11 samples of blood and urine were taken in the mornings for the determination of normal values. Since the dogs were allowed exercise for a short period each day, the urinary values are not complete. During the injection periods the growth hormone was injected in the morning and the first meal was given. The second injection and meal were given 8 hours later. In the first injection period of 7 days the growth hormone caused, in all the dogs, diabetes that appeared about three days after the first injection. The diabetes was so severe in dog F that death in diabetic coma occurred about 20 hours after the last of these injections. A rest of 30 days followed for the remaining 3 dogs, during which the signs of diabetes disappeared. Injections of growth hormone were then given again for 6 to 8 days, and the animals were sacrificed on the day after the last injection. During the preliminary and the first injection periods the daily diet consisted of 300 gm. ground horse muscle, 100 gm. Purina dog checkers and 40 gm. sucrose, this being divided into 2 meals. In the resting and the second injection periods this diet was halved in amount.

The five control dogs were also adult males maintained under the same conditions. They were given 3.5 mg. of bovine plasma albumin[6] (BPA) per kg. of body weight per day as two subcutaneous injections, instead of growth hormone. They were given the full diet, and as much blood was withdrawn each day as was taken from the test animals.

Throughout these experiments care was taken to minimize the factors tending to produce infection. The site of injection was shaved, cleaned and painted with iodine, and the solution of growth hormone was given subcutaneously from a sterile syringe and needle. The dogs were examined daily by two observers, but no signs of local infection were found. On a few occasions, a slight oedema or induration could be felt on the day following

[5] We are much indebted to Professor H. M. Evans and Dr. C. W. Asling who tested the biological activity of D21P in young hypophysectomized rats given a total dose of 2 mg. in 4 days. The results were as follows:— TSH+ to ++; ACTH± to +; lactogenic hormone++ to +++; interstitial cell-stimulating hormone+; and follicle-stimulating hormone−.

[6] Armour Laboratories, Chicago.

an injection, but these disappeared after 1 to 2 days. At autopsy no local infection was found on histological examination. Blood cultures from the dogs were examined at intervals during these injections in the Department of Bacteriology, but no growth was found. Solutions of the growth hormone for injection were also cultured, with negative results.

RESULTS

Blood sugar, etc.

The administration of the bovine growth preparation D21P in dosage of 3.5 mg./kg. of body weight per day to the 4 intact dogs produced marked hyperglycaemia and glycosuria during the first 7-day period of injections (Fig. 1). In all cases these increases in blood sugar were well maintained during the injections. Glycosuria, polyuria and polydipsia occurred in all these dogs (Fig. 1), and ketonuria appeared, becoming more marked as the injections continued. The blood plasmas in dogs F, M and P were visibly lipaemic on the 2nd, 3rd and 4th days after the first injection. While the diabetes elicited by growth hormone was severe in all cases there were gradations in the severity, the descending order being that in dogs F, M, P and R.

Certain changes in the general condition of these dogs occurred during the injections of the purified growth hormone. After 1 or 2 days of diabetes their usual high spirits were lost and irritability followed. Towards the end of the injections lethargy became pronounced. In all cases the rate of respiration was much increased, and the dogs panted almost continuously. The rectal temperatures on the third day were 38.5, 37.2, 38.0 and 38.2° C. in dogs F, P, M and R respectively. Vomiting occurred in all cases, being most severe in dog F and least in dog R. This occurred particularly after meals. Loss of appetite and finally refusal of some of the food followed. The high intake of water continued, however, with vomiting of water and mucus. Towards the end of the injection period the appearance of tarry stools suggested haemorrhage from the upper alimentary tract. Persistent tremors were noted in the fore and hind limbs of dogs F and P. In these dogs particularly, dehydration was evident.

These signs were most pronounced in dog F and, on the morning of the 8th day from the first injection, he was found in a comatose condition. Despite the administration of insulin and of glucose in saline intravenously, he succumbed about mid-day in diabetic acidosis.

Following the injections, the blood sugar returned to normal within 2 days in 2 cases, but in dog M it was still very high after 3 days, and 40 gm. of sugar were excreted during the third day. By the 4th day of rest the values were normal. The lethargy moderated and appetite returned, and

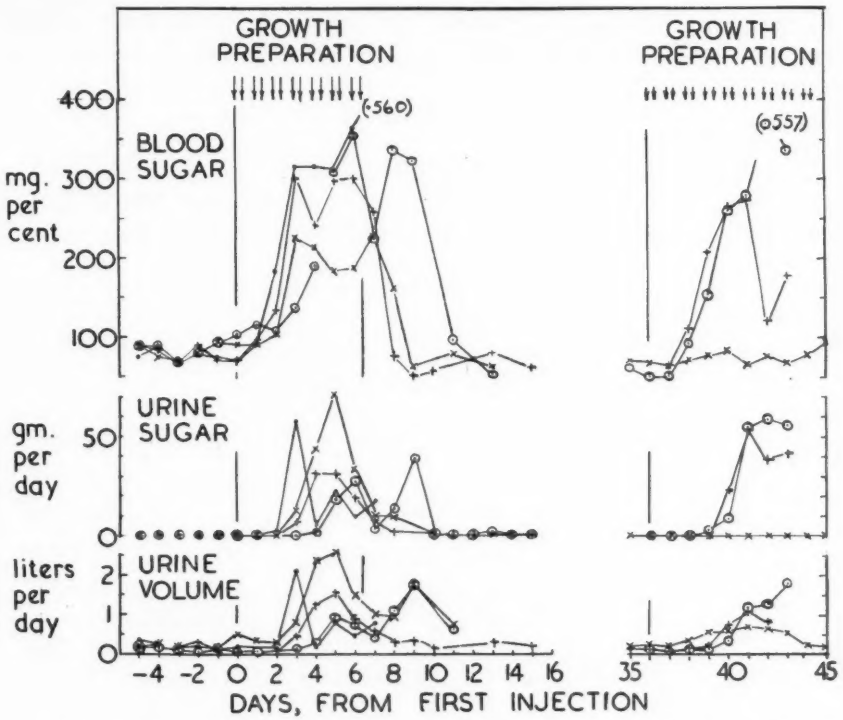

FIG. 1. The intact dogs were given growth preparation D21P at the times indicated by the arrows. The dosage was 1.75 mg. per kg. per injection from days 0 to 6.5 inclusive, and 1 mg. per kg. from days 36 to 44.5, there being 2 injections per day. Dog: F, ·; P, +; M, ⊙; R, ×.

by the 4th to 6th day of rest the dogs appeared to have recovered their former vigour. During this resting period, and until the end of the experiment, the diet was reduced to one-half the former amount.

Growth hormone D21P was again administered (second injection period) to the 3 dogs in the reduced dosage of 2 mg./kg. per day. These reductions in dosage and diet were made because of the severity of the diabetes in the first series of injections. Hyperglycaemia occurred in dogs M and P from the 3rd day after the first of these injections (Fig. 1). Sugar was excreted and the urine volume was high while ketonuria occurred in dog M. Although the signs of diabetes were not as intense as during the first series of injections, they were nevertheless severe. Lethargy, panting, some failure of appetite and vomiting were observed. In dog R, however, the blood sugar did not rise appreciably, and the urine remained sugar-free. He ap-

peared to be only slightly depressed by the injections, and was evidently more resistant to the diabetogenic effects of the growth hormone than the other dogs.

Excretion of nitrogen

Since a small proportion of the urine was lost daily, the values for the nitrogen and some constituents of the urine during the initial and first injection period, shown in Table 1, are only roughly comparable. The oc-

TABLE 1. NITROGENOUS CONSTITUENTS OF URINE

The average daily values of total nitrogen and of certain nitrogenous substances of the urine, excreted prior to, and during the injections of growth preparation D21P in dosage of 3.5 mg. per kg. per day, are shown. The daily values during 6 days in each of these periods were averaged. A small amount of urine was lost each day, but it is thought that the values in the 2 periods are comparative.

Dog	Period	Urine, average daily values per kg. of body weight			
		Total N. mg.	Urea N. mg.	Creatinine mg.	Uric acid mg.
F	Pre-injection	830	241	0.78	3.1
P		755	568	0.32	2.7
M		743	525	0.45	2.1
R		767	276	0.75	2.9
Means		774	402	0.57	2.7
F	During injection of growth hormone	561	150	0.57	2.6
P		353	206	0.89	7.9
M		392	53	0.56	3.2
R		539	342	0.98	8.4
Means		461	188	0.75	5.5

currence of vomiting and decrease in food intake in some cases further decrease the significance of the values. When growth hormone was given the excretion of total nitrogen and urea decreased, while that of uric acid increased in these dogs.

Extractable insulin of the pancreas

At the end of the second series of injections the extractable insulin content of the pancreas was much less in these animals given growth hormone than in the control dogs. In dogs F, P and M the amount was 1 to 2% of the average for 5 control dogs, and in dog R it was about 20% of the control value (cf Fig. 1 and Table 2).

Erythrocyte sedimentation rate

The administration of the growth hormone increased the erythrocyte sedimentation rate (ESR) in all cases (Fig. 2). During the first series of injections the values rose from 1 to 4 mm./hour to about 40 mm./hour. In

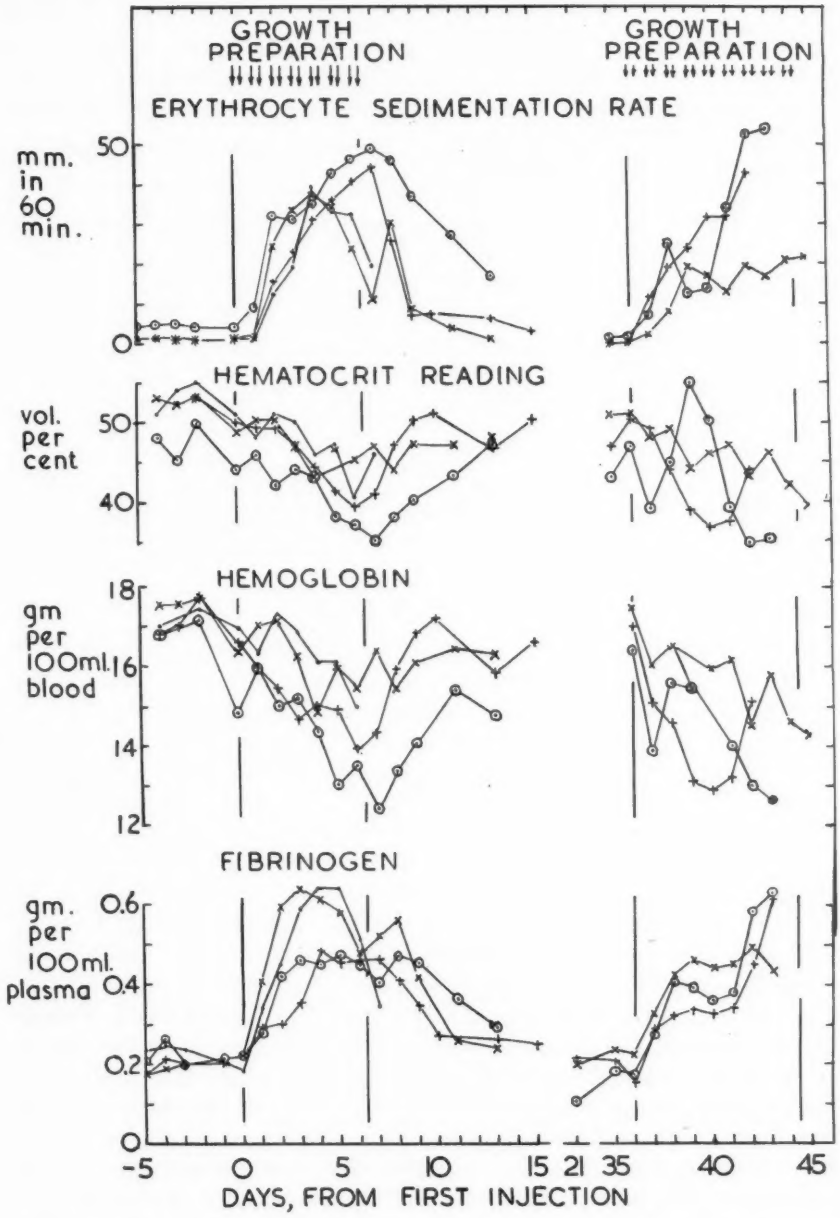

FIG. 2. The dogs were given injections of growth hormone (D21P). Each arrow indicates the injection of 1.75 mg. per kg. from days 0 to 6.5 inclusive, and 1 mg. per kg. from days 36 to 44.5, there being 2 injections per day.

TABLE 2. EFFECTS OF GROWTH HORMONE ON THE EXTRACTABLE INSULIN OF THE PANCREAS

Growth hormone D21P was administered to the four test dogs for 7 days in dosage of 3.5 mg. per kg. body weight per day. The extractable insulin of the pancreas of dog F was then determined. After a rest of 30 days, growth hormone was again given to the other three test dogs in dosage of 2 mg. per kg. for 7 to 9 days, and the extractable insulin contents of their pancreases were determined.

The control dogs were injected with 3.5 mg. BPA per kg. body weight for 7 days before extracting the insulin of the pancreases.

Injections	Dog	Body weight kg.	Pancreas weight per kg. body wt. gm.	Extractable insulin of the pancreas		No. mice injected
				per gm. pancreas units	per kg. body wt. units	
Growth hormone D21P	F	12.0	2.45	0.066	0.18	66
Growth hormone D21P	P	9.05	2.30	0.039	0.09	24
Growth hormone D21P	M	9.15	2.95	0.083	0.25	48
Growth hormone D21P	R	12.5	1.87	0.83	1.65	78
Test dogs, Means			2.39			
Bovine plasma albumin	N1	12.5	1.42	5.24	7.38	42
Bovine plasma albumin	N2	13.7	1.59	3.73	5.90	66
Bovine plasma albumin	N3	8.5	1.65	4.80	7.9	42
Bovine plasma albumin	N4	8.6	1.74	6.44	11.2	42
Bovine plasma albumin	N5	8.5	2.75	2.41	6.65	42
Controls, Means			1.83	4.52	7.81	

the resting period the values returned to normal in 3 days, except for that of dog M which was still above the pre-injection level after 7 days. The hyperglycaemia and glycosuria also persisted longer in this dog after the injections were stopped. In the second period of injections, with the lower dose of growth hormone, the ESR rose again in the 3 dogs.

Haematocrit readings, haemoglobin and red cell counts

The volume per cent of packed erythrocytes decreased during both periods of administration of the growth hormone in these dogs (Fig. 2). There were variations from day to day, and, on occasion, in dogs F and M the haematocrit readings during the injections rose to the pre-injection levels. In the resting period the haematocrit values returned to normal, more slowly in dog M. Determinations of the effects of dilution of blood cells by plasma showed that the effects of the growth hormone on the ESR cannot be accounted for by these reductions in the haematocrit values.

The concentration of haemoglobin in the blood was also decreased in all cases during both periods when growth hormone was administered (Fig. 2). Red cell counts were not made during the first series of injections but were found to be decreased during the second series. In general, the haematocrit readings, haemoglobin concentrations and red cell counts varied in unison.

The reticulocyte counts were fairly uniform in the dogs M, P and R, ranging from 0.48 to 1.12 per 100 erythrocytes. The administration of the growth hormone preparation did not alter the number of the reticulocytes significantly.

Blood plasma volume and cell volume

The plasma volume, per kg. body weight, increased in each dog during both periods of injection of growth hormone. During the resting period the values returned to normal. These increases in plasma volume ranged from 10 to 50% above the values prior to injection of growth hormone. The average plasma volumes for all the dogs, in each period, show that the increases when the growth hormone was given are statistically significant as compared to the preliminary and resting period values (Table 3). The *t* and probability (P) values are calculated according to Fisher and Yates (1938).

TABLE 3. VOLUMES OF BLOOD CELLS AND PLASMA

Growth hormone, D21P, was given to the 4 dogs in dosage of 3.5 mg. per kg. body weight per day during the first injection period of 7 days, and in dosage of 2 mg. to 3 dogs in the second injection period. The average red cell volumes, and the plasma volumes, per kg. body weight, are shown with their standard errors.

Treatment		Total No. of determinations	Volume of red blood cells	Volume of plasma
Period	Days		ml. per kg. body weight	
Preliminary	30	10	48.5 ± 2.3	46.3 ± 1.77
GH injections	7	5	46.1 ± 3.3	57.4 ± 1.90
	t		0.59	3.89
	P		0.60	0.007
Resting	30	7	46.2 ± 1.9	46.6 ± 2.16
GH injections	7–9	4	45.2 ± 8.4	62.1 ± 3.36
	t		0.16	4.10
	P		0.88	0.006

The volume of blood cells, per kg. body weight, however, was not changed significantly by the administration of growth hormone (Table 3). The growth hormone thus increased the blood volume by increasing the volume of the plasma.

In each of 2 control dogs, the injections of BPA did not alter the plasma volume nor the red cell volume (42.7 and 50.8 ml. plasma per kg. body weight before; and 42.2 and 51.1 ml. after injection in control dogs 1 and 2 respectively). In these control dogs also the haematocrit readings, haemoglobin concentrations and the ESR did not change appreciably (Fig. 7).

It may be concluded therefore that the reductions in the volumes per cent of red cells, in haemoglobin concentrations and in red cell counts that

occur when growth hormone is given are caused by dilution of the cells with the increased volume of plasma, and are not due to decreased amounts of red cells or haemoglobin in the blood.

Plasma proteins, concentrations and amounts

When growth hormone was given, the average concentration of total protein in the plasma, determined as nitrogen, was not significantly altered (Table 4). However when the total protein was estimated by the method of Phillips *et al.* (1950) the specific gravity of the plasma increased within the injection periods by 0.002 to 0.004, equivalent to increases in

TABLE 4. PLASMA PROTEIN CONCENTRATIONS

The mean concentrations of protein in the blood plasma for all the dogs in each period are given.

Period	Plasma proteins, means, gm. per 100 ml. plasma				
	Total	Albumin	Globulin	A/G	Fibrinogen
Preinjection	5.93	3.30	2.48	1.33	0.19
GH injections, 1st period	6.09	2.83	2.82	1.00	0.47
t	1.83	4	2.66	3	6
P	0.9	<0.001	0.01	0.01	<0.001
Resting	6.03	3.35	2.48	1.35	0.20
GH injections, 2nd period	6.07	3.25	2.43	1.33.	0.38
t		1.7		0.07	6
P		0.1		1	<0.001

protein of 0.7 to 1.5 gm.%. The considerable variance of the protein concentrations, determined as nitrogen, may have obscured a possible increase in total plasma protein.

During the first period of injection of growth hormone the concentration of plasma albumin decreased, while that of globulins (other than fibrinogen) increased, as compared to the pre-injection concentrations. The A/G ratio decreased in the injection period (Table 4). These decreases in albumin and increases in globulin concentrations are statistically significant. During the second period of injections of growth hormone the albumin and globulin values were not changed. This lessening of the responses is probably related to the reductions in diet and in dosage during the resting period and the second series of injections.

The growth hormone caused remarkable increases in the fibrinogen concentrations of the plasma in each dog during both periods of injection (Fig. 2). The fibrinogen concentrations were above the initial levels 24 hours after the first injection and the increase continued to values that

were double the initial values. On ceasing the injections the fibrinogen concentrations returned gradually towards the initial levels during about 7 days in the resting period. During the first and second injection periods the average fibrinogen concentrations were 2.5 and 1.9 times the pre-injection values respectively (Table 4). The concurrent determination of the rate of fibrinogenolysis required that the fibrinogen in replicate samples of diluted plasma be determined after 1, 3 and 6 hours of incubation. The rates of fibrinogenolysis proved to be low, and the determinations for each dog agreed closely, showing that these values are consistent.

In the control dogs, the injections of BPA did not alter the concentrations of total protein, albumin, globulin or fibrinogen appreciably (Fig. 7).

TABLE 5. AMOUNTS OF PROTEINS IN THE PLASMA

The average amounts of proteins in the plasma, per kg. body weight, for all the dogs in each period are shown with the standard errors.

Treatment		Determinations Total No.	Plasma protein, average amounts, gm. per kg. body weight			
Period	Days		Total	Albumin	Globulin	Fibrinogen
Preliminary	30	10	2.66 ±0.107	1.34 ±0.067	1.25 ±0.120	0.085 ±0.00709
GH injection, first	7	5	3.51 ±0.185	1.44 ±0.144	1.76 ±0.104	0.262 ±0.00206
		t	4.28	0.82	2.72	10.2
		P	0.001	0.40	0.018	<0.001
Resting	30	7	2.74 ±0.284	1.51 ±0.081	1.13 ±0.071	0.087 ±0.0117
GH injection, second	7–9	4	4.36 ±0.39	2.13 ±0.119	1.44 ±0.124	0.290 ±0.044
		t	5.27	4.46	2.36	5.66
		P	<0.001	0.002	0.045	<0.001

The amount of protein in the plasma was calculated by multiplying the volume of the plasma by the concentration of protein. The quantity of total protein in the plasma, per kg. body weight, was increased in each dog by the growth hormone during both periods of administration. The average amounts of total protein, in all the dogs, in the two periods when growth hormone was given were significantly greater than in the preliminary and resting periods (Table 5). This was due chiefly to the increased volume of plasma.

The results do not permit of a definite conclusion regarding the effect of growth hormone on the amount of albumin in the plasma. The average amount was not significantly changed during the first injection period, but was increased during the second injection period (Table 5). There was a progressive increase in the average amount of albumin from the preliminary to the second injection period. The growth hormone increased signifi-

cantly the average amount of globulin in the circulating blood (Table 5). This was observed in the average values for each dog, and during both periods of administration of growth hormone.

The amount of fibrinogen in the plasma was considerably increased, in each dog, by the growth hormone during both periods of administration. These increases in the individual dogs were from 2 to 8-fold the amounts in the preliminary and resting periods. The average amounts of fibrinogen, for all the dogs, were increased 3-fold by the growth hormone injections, this increase being highly significant (Table 5).

In two control dogs given BPA for 7 days the amounts of total protein and the fractions thereof in the plasma did not change appreciably.

Viscosities of blood and plasma

The administration of the growth hormone usually decreased the viscosity of the blood, as determined by the capillary tube method, in these dogs. This effect may be explained by the reduction of the volume of blood cells relative to plasma. The viscosity of the plasma increased slightly when the growth hormone was given. This is probably due to increased concentrations of fibrinogen and other globulins of high molecular weight in the plasma of the dogs given growth hormone.

Clotting time and prothrombin time

The clotting time of the blood was definitely decreased in all the dogs by the growth preparation, during both periods of administration (Fig. 3). The prothrombin time of the plasma, determined by the one-stage method of Quick (1938) was also rapidly decreased by the growth hormone in all cases during both periods of injection. These decreases in clotting and prothrombin times were prompt in appearance and were progressive.

Fibrinogenolysis

Determinations of the rates of fibrinogenolysis showed that, prior to the injections, the average amount of fibrinogen which disappeared from the samples of diluted plasma at pH 7.4 and 37° C. in 6 hours was negligible. (In a total of 23 determinations, 10 showed a change of ± 0.01 gm. fibrinogen; 9 showed ± 0.02; 2 showed ± 0.03 and 2 showed -0.05.) During the administration of growth hormone the amount of fibrinogen that disappeared, on incubation of the diluted plasma under the standard conditions, increased. (The average decreases for 4 dogs, on days 1 to 7 were 0.03, 0.05, 0.06, 0.05, 0.02, 0.03 and 0.02 gm./100 ml. plasma.) The values remained elevated for some days in the resting period (daily average decreases for 3 dogs were 0.06, 0.03, 0.04, 0.03 and 0.02). However, although

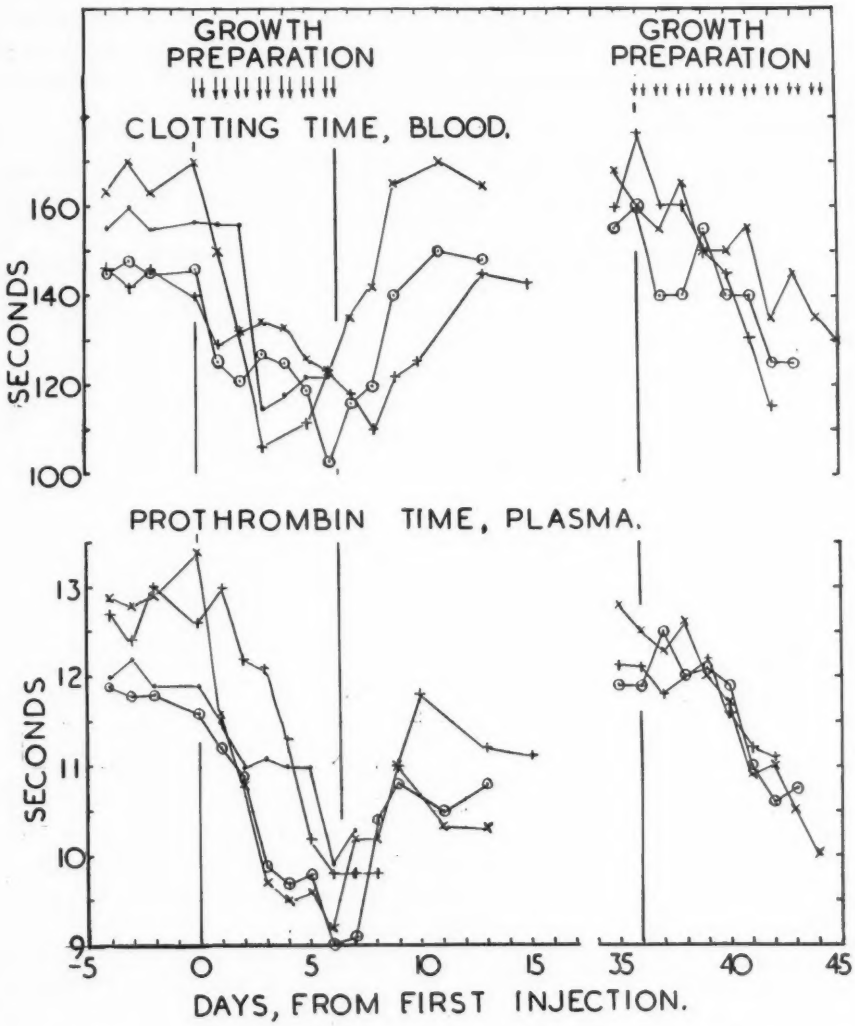

FIG. 3. The clotting time of the blood and the prothrombin time of the plasma of dogs given growth hormone. Each arrow indicates the injection of 1.75 mg. growth hormone preparation D21P per kg. body weight from days 0 to 6.5 inclusive, and 1 mg. per kg. from days 36 to 44.5, there being 2 injections per day. Dog: F, ·; P, +; M, ⊙; R, ×.

the amount of fibrinogen which disappeared from the plasma during incubation was increased after giving the growth hormone, that lysed per gm. of fibrinogen was not noticeably altered. A few determinations of the rates of fibrinolysis gave similar results during the second series of injections.

Plasma non-protein nitrogen

In 3 dogs (F, P and R) a fairly consistent pattern of change in the plasma non-protein nitrogen (N.P.N.) was found. Following the administration of

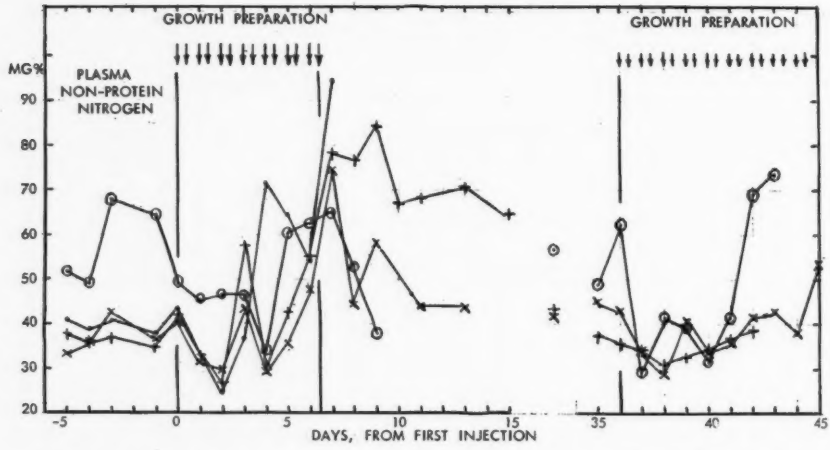

FIG. 4. The intact dogs (F, ·; P, +; M, ⊙; and R, ×) were injected, as indicated by the arrows, with 1.75 mg. growth hormone preparation, D21P, per kg. from day 0 to 6.5 inclusive; and 1 mg. per kg. from day 36 to 44.5.

growth hormone in these cases, the N.P.N. was decreased for 2 days, but thereafter was markedly increased and was variable (Fig. 4). In dog R the values remained high for 8 days after the first series of injections. On reinjection a similar though less evident trend was seen.

In dog M the values both before and during the injections were high and variable. However, in this case also, on giving growth hormone lower values of plasma N.P.N. were found for 4 days, followed by an increase.

Leucocytes

On administering growth hormone a considerable increase in the total leucocytes of the blood was found in all dogs (Fig. 5). These increases were usually noticeable 1 day after the first injection and continued as long as the injections were given. In the resting period the leucocyte counts de-

creased rapidly, but were still above the initial levels after 7 days. By the 24th day of rest the values had returned to normal. On readministration of the growth hormone at the reduced dosage, the leucocytes again increased for 7 to 9 days.

Differential counts showed that this leucocytosis was due chiefly to marked increases in the neutrophil granulocytes, as can be seen by inspection of Figure 5. In all cases, during both periods of administration of the growth hormone, the increased numbers of these cells accounted for the major part of the increase in total leucocytes. The neutrophil "stab" cells, whose nuclei appeared narrow, ribbon-like and unsegmented, were counted separately during the second series of injections and were found to be increased also by the growth hormone (Fig. 5).

When the growth preparation was given, the lymphocytes, monocytes, eosinophils and basophils were decreased in proportion to total leucocytes, i.e., per 100 white cells. However, when the values were expressed as the actual number of cells per cu. mm. blood, the numbers of lymphocytes, eosinophils and basophils were not altered by the injections. The numbers of monocytes increased slightly (Fig. 6).

In control dogs, the leucocytes were not changed during the administration of BPA in one case, but in the other a slight increase occurred (Fig. 7).

The cells in smears of bone marrow from the ribs were differentiated and counted in these test and control dogs. These examinations did not reveal changes in the frequency distribution of the various cell types.

Sludging of blood

The dogs P, M and R were anaesthetized (intravenous nembutal) after receiving the second series of injections of growth hormone for 6, 7 and 9 days respectively. The flow of blood through the vessels of the mesentery was then observed by transmitted light, which was filtered through 9 cm. of water and a layer of heat-absorbing glass. The magnification was about 400. The method of examination is of course not quantitative. From independent observations, the 4 observers were in agreement that in these 3 dogs there was evidence of "sludging" of blood as it flowed through the small vessels, as compared to the flow in 2 control dogs (N1 and N2).

Organ weights and lipid contents

Immediately following visualization of the mesentery in dogs P, M and R, the femoral artery was cannulated and blood was withdrawn until the flow stopped. In the dogs given growth hormone the liver was much enlarged, the average weight being more than double that of the 5 control animals (Table 6). The dry fat-free solids, the water and the fat in the liver,

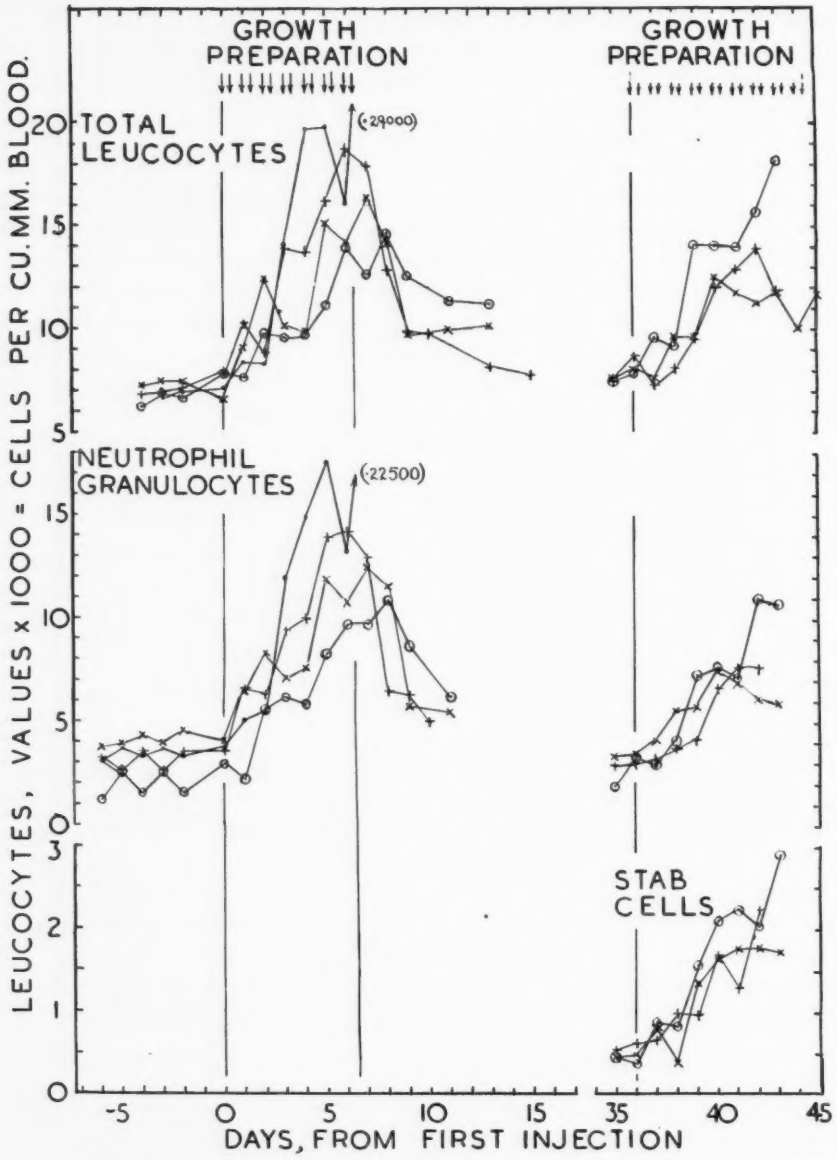

FIG. 5. The intact dogs were given growth preparation D21P at the times indicated. The dosage was 1.75 mg. per kg. body weight per injection from days 0 to 6.5 inclusive, and 1 mg. per kg. from days 36 to 44.5, there being 2 injections per day. Dog: F, ·; P, +; M, ⊙; R, ×.

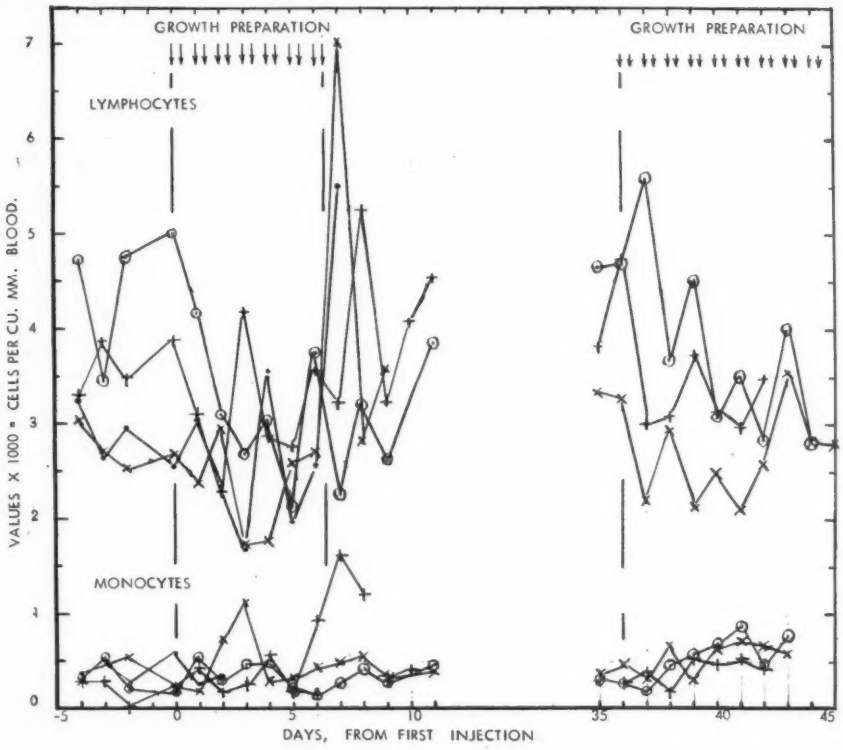

FIG. 6. The intact dogs (F, ·; P, +; M, ⊙; and R, ×) were injected, as indicated by the arrows, with 1.75 mg. growth hormone preparation D21P per kg. from days 0 to 6.5 inclusive, and 1 mg. per kg. from days 36 to 44.5.

per 100 gm. of body weight, all were increased in the dogs given growth hormone. The fat content of the liver varied considerably, being greatest in M and least in R. The magnitude of the changes in blood sugar (Fig. 1), extractable insulin of the pancreas (Table 2), ESR (Fig. 2), and neutrophil granulocytes and stab cells (Fig. 5) followed the same order in these dogs.

The liver fat was not determined in the dog F that succumbed in diabetic coma at the end of the first series of injections. In this case, however, the liver weight (65.8 gm./kg. body weight) was greater than in any of the other test dogs (cf. Table 6). The organ was obviously very fatty, and histological examinations indicated that it contained more fat than in any of the other cases. The kidney weight (7.00 gm./kg. body weight) was also greater than in any of the other dogs which later were given the second series of injections of growth hormone.

TABLE 6. ORGAN WEIGHTS AND LIPID CONTENTS

Growth hormone D21P was administered to the intact dogs of the test group, and BPA was given to the controls as described in table 2.

Injections	Dog	Body wt. kg.	Liver			Kidney			Heart	
			Weight	Dry fat-free solids	Total lipid	Weight	Dry fat-free solids	Total lipid	Dry fat-free solids	Total lipid
			gm. per kg. body weight						gm. per 100 gm. fresh tissue	
Growth hormone, D21P	P	9.04	41.5	8.88	3.50	6.76	0.77	0.22	19.1	4.3
Growth hormone, D21P	M	9.15	56.0	8.73	14.0	6.50	1.00	0.37	19.4	6.3
Growth hormone, D21P	R	12.5	41.3	10.0	2.20	6.80	1.10	0.29	19.0	4.7
Means, test dogs			51.1	9.2		6.77	0.96	0.29	19.2	5.1
Bovine plasma albumin	N1	12.5	22.7	5.43	1.70	5.30	0.82	0.38	19.4	5.0
Bovine plasma albumin	N2	13.7	28.3	7.10	1.50	4.93	0.81	0.31	19.1	4.2
Bovine plasma albumin	N3	8.5	21.2	5.53	1.42				21.8	4.0
Bovine plasma albumin	N4	8.6	25.6	6.57	1.49	4.91	0.99	0.23	18.6	3.4
Bovine plasma albumin	N5	8.5	21.3	5.68	1.33	4.64	0.89	0.20	21.5	4.0
Means, controls			23.8	6.06	1.49	4.95	0.88	0.25	20.1	4.1

In the 3 test dogs given the second series of injections of growth hormone, the kidney weights were above those of the controls (Table 6). It is not clear from the values as to what, if any, were the effects of the injections on the fat-free solids or on the lipids of the kidney. There is some indication that the former were increased. The injections of growth hormone did not alter the lipid content of the myocardium significantly (Table 6).

DISCUSSION

One of the most prominent features of this investigation is the great intensity of the diabetes elicited in these intact dogs by the relatively small doses of highly purified growth hormone. The response in dogs is not a quantitative measure of diabetogenic activity, but it is clear that the responses have been more intense with the preparations of higher growth activity (Campbell and Davidson). The degree of contamination of the growth hormone preparation with ACTH, TSH, prolactin and gonadotrophins is of a low order. The hyperglycaemic and glycosuric effects of ACTH in dogs (Campbell, Davidson, Snair and Lei, 1950) are much less than those of growth preparations, and it is not expected that this contamination with ACTH would have a noticeable effect on blood sugar. According to Young (1938), TSH and prolactin are not diabetogenic in dogs. The electrophoretic and ultracentrifugal analyses of the growth preparation used in the present study show that the growth hormone is present, with about 5% of material which has higher electrical mobility, and is of higher molecular weight. Raben and Westermeyer (1952) claim that a growth hormone fraction from hog pituitary glands can be prepared free of diabetogenic activity. To the contrary we find that in preparations of

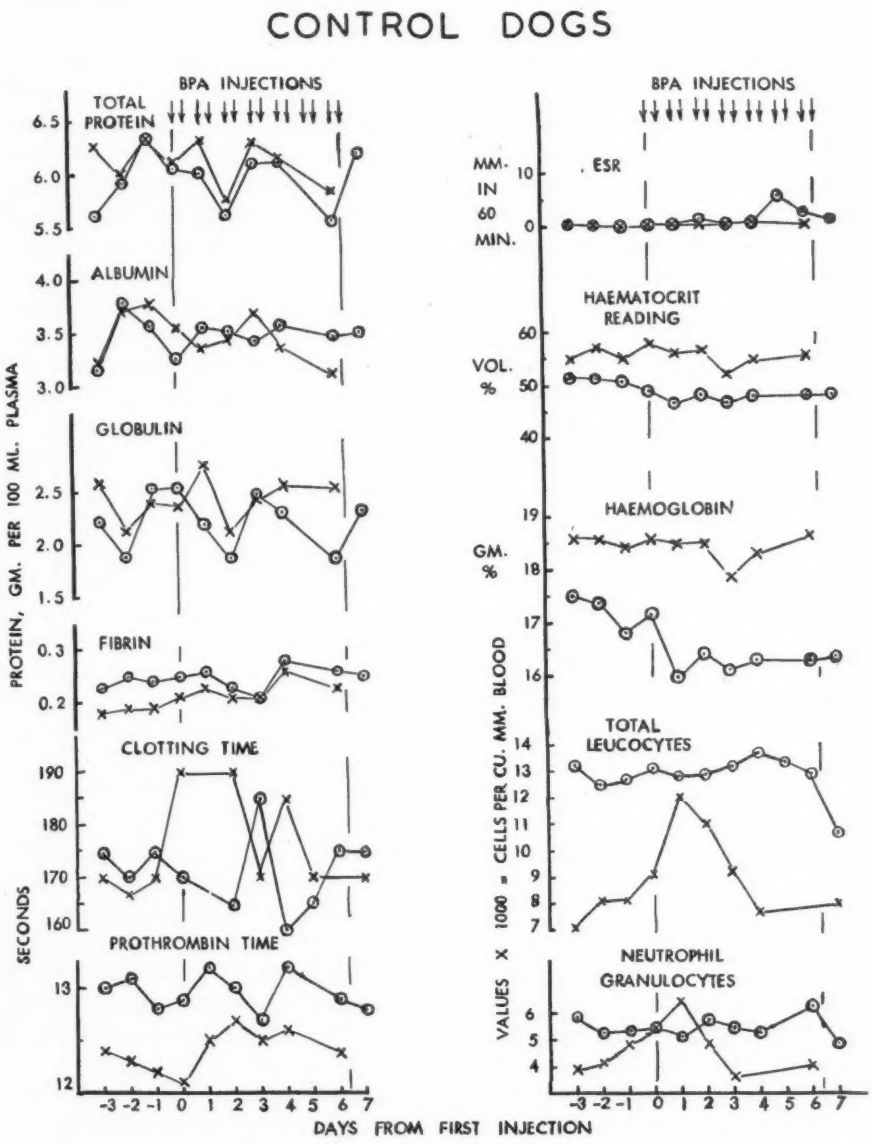

FIG. 7. Normal, control, dogs given injections of 1.75 mg. bovine plasma albumin (BPA) per kg. body weight, as indicated by the arrows. Dog: N1, ×; N2, ⊙.

bovine growth hormone the growth activity parallels the diabetogenic activity (Campbell *et al.*, 1950). If it is postulated that the preparation contains both a growth-promoting and a diabetogenic substance, then the two hypothetical factors must have the same electrophoretic mobilities at pH 4 and 9, and also have almost identical molecular weights; or, alternatively, the diabetogenic factor must produce these effects in dosage of 5% of that used in these experiments together with 95% of the growth hormone. These possibilities seem to impose peculiarly stringent requirements. The present evidence indicates therefore that a single protein molecule from bovine anterior pituitary glands possesses both growth-promoting activity in hypophysectomized rats and diabetogenic activity in dogs. Reid (1952) also finds correspondence between growth and diabetogenic activities.

Changes which occur in idiohypophyseal diabetes produced by crude or partially purified anterior pituitary extracts in dogs have been described by Houssay (1942) and others. Many of these changes (hyperglycaemia, glycosuria, polyuria, polydipsia, ketonuria, lipaemia, reduced amount of insulin extractable from the pancreas, increase in liver weight and lipid content) are also produced by the administration of the highly purified growth hormone preparation used in the present investigation. There is thus little support for the postulate that the diabetogenic factor is necessarily of multiple nature. It is of course to be expected that when crude extracts are given, other pituitary principles may alter the action of the growth hormone.

Certain relations of the blood sugar level to the various effects produced by the growth preparation emerge from these results. The dog R was most resistant to the hyperglycaemic effect of the growth hormone in the first series of injections, and in the second series, with a lesser dose and diet, no fasting hyperglycaemia, and no glycosuria or ketonuria were found. Yet in this dog, as in the others during the second series of injections, the plasma volume and the amounts of plasma protein and of fibrinogen were increased (Tables 3 and 5), the haematocrit readings and haemoglobin concentrations decreased (Fig. 2), the ESR increased (Fig. 2), the clotting times and the prothrombin times decreased (Fig. 3) and neutrophilia occurred (Fig. 5). These changes induced by the administration of the growth hormone are therefore not necessarily dependent on the sugar content of the blood, nor on ketosis.

It is also evident that the decrease in the insulin content of the pancreas caused by the growth hormone is not dependent on blood sugar level alone. In one of the dogs (R), for example, no hyperglycaemia and no glycosuria occurred during the second period of injection of growth hormone, yet the amount of insulin extractable from the pancreas was only one-fifth of that found in control dogs (Table 2). Best, Campbell and Haist (1939) noted

that there was, in a general sense, an inverse relation between the rise in blood sugar and the fall in the insulin content of the pancreas in dogs given a diabetogenic pituitary fraction. It was not claimed, however, that the fall in the insulin content was caused by the increase in blood sugar alone, for in some cases the insulin content was definitely reduced after 1 to 4 days of injection, while the fasting blood sugar did not exceed 124 mg.%. The effects of hyperglycaemia, produced by the parenteral administration of glucose, on the islet cells of experimental animals have been described by Woerner (1938), Houssay *et al.* (1942) and Dohan and Lukens (1948).

Nevertheless the magnitude of the increase in blood sugar appears to be related to the intensity of the effects of growth hormone on the extractable insulin of the pancreas (Table 2), on the leucocytes of the blood (Fig. 5) and on the lipid content of the liver (Table 6). The relation of the blood sugar level to the other effects of the growth hormone is not consistent in all cases. The evidence indicates that all these changes are caused by the growth hormone. The metabolic characteristics of the individual dogs, the dosage of growth hormone and the other experimental conditions must determine the degrees, and the times of appearance, of these changes. Thus, when growth hormone is given to dogs, the level of sugar in the blood may be regarded as an index, not always reliable, of the intensity of the effects of the hormone. It is also clear that other significant changes may occur in the absence of obvious effect on blood or urine sugar.

The plasma volume and the amount of total protein in the plasma were increased by the growth hormone. It is suggested that this increase in volume may be dependent upon the increased quantity of protein in the plasma requiring an increased volume of water. This explanation is supported by the finding of Beattie and Collard (1942) that repeated transfusions of plasma to cats increased both the concentration of protein in, and the volume of the plasma.

The increase in the amount of total protein in the blood plasma caused by growth hormone was due chiefly to the increase in proteins other than fibrinogen. Although the globulins increased in concentration, the important factor in relation to amount was the increase in plasma volume. The most striking effect of growth hormone on the plasma proteins was on the concentration and amount of fibrinogen, which increased several fold over the pre-injection levels. These results therefore show that the injection of growth hormone increased the rate of addition of protein to the blood relative to the rate of removal. Whether this was due to release of stored protein, increased rate of synthesis or decreased rate of removal is at present unknown. Some experimental evidence bearing on this problem does exist, however.

Gaebler, Bartlett and Sweeney (1951) demonstrated gains in body

weight and nitrogen storage in dogs given growth hormone. In the present study the dogs gained weight during the first series of injections, except dog F which soon became severely diabetic. The body weights also increased slightly during the second series of injections, although the dietary intake was low. The injections reduced the excretion of nitrogen and of urea, while the plasma N.P.N. was reduced for a few days after which the initial values were exceeded. In nephrectomized rats given a casein hydrolysate, Russell and Cappiello (1949) found that growth hormone increased the rate of uptake of amino acids by the tissues and decreased the amount of urea formed. The studies of Young (1945) indicate that diabetogenic anterior pituitary extracts increase the amount of protein in the body by increasing the rate of synthesis and also by decreasing the rate of utilization of protein. These previous results suggest that purified growth hormone may increase the amounts of plasma protein by stimulating increased synthesis, and probably also decreased utilization, of protein.

According to Madden and Whipple (1940) fibrinogen is produced by the cells of the liver. In the dogs given growth hormone the liver increased in weight and in fat-free solids, which consist chiefly of protein. The increase of fibrinogen in the circulating blood of dogs given growth hormone cannot well be explained as due merely to release of stored (preformed) fibrinogen or fibrinogen precursor from the liver. It is most likely therefore that the growth hormone increased the rate of synthesis of fibrinogen by the liver. It is of interest that the quantity of plasma protein increased while sugar was being lost in the urine.

The total quantity of red cells in the blood was usually not definitely altered by the administration of growth hormone. The decreases in the volumes per cent of packed erythrocytes, in erythrocyte counts and in haemoglobin concentrations can be attributed therefore to dilution of the cells with the increased volume of plasma. The decrease in viscosity of the blood can also be attributed to the reduction of cells per unit volume of blood. The increase in plasma viscosity is probably due to the increased proportions of proteins of high molecular weight in the plasma, as shown by the increase in fibrinogen and the reduced A/G ratios.

The rapid ESR in the dogs given growth hormone is most likely chiefly due to the increased concentrations of fibrinogen in the plasma. This protein is particularly effective in producing aggregation of cells, thereby increasing the rate of sedimentation (Wintrobe, 1946). The reduction of the haematocrit readings can account for only a small part of the total effect on ESR. The indications of "sludging" of blood as it flowed through the small vessels of the mesentery, in the dogs given growth hormone, are probably chiefly due to the increased tendency of the cells to aggregate.

The decreased clotting times in the dogs given growth hormone indicate an activation of the clotting system. Although the prothrombin times also decreased, the relation of this observation to the clotting system apparently cannot yet be interpreted (Brambel, 1950). However, the indications of increased protein synthesis in the liver may prove to be of interest in regard to the shortened prothrombin times. It may be noted that, in certain cases of diabetes mellitus, Giannco and Marraza (1948) found that the fibrinogen and prothrombin of the blood were above normal.

In view of the low to very small amounts of insulin that could be extracted from the pancreas after 7 days of injection of growth hormone in dog F, and after the second series of injections in the other 3 dogs, it would seem likely that at these stages of the experiment the secretion of insulin was less than normal. The severity of the diabetes at this stage also supports this conclusion. However, Campbell, Munroe, Hausler and Davidson (1953) deduced that, at earlier stages of administration, the growth hormone may possibly cause an increased rate of secretion of insulin from the pancreas. The explanation is advanced therefore that the decrease in the extractable insulin of the pancreas and the diabetes in these dogs is due to overwork and partial exhaustion of the insulin-secreting beta cells of the islets of Langerhans. Haist, Campbell and Best (1940) suggested previously that metahypophysial or permanent pituitary diabetes, produced in dogs by a diabetogenic pituitary fraction, was caused by overwork of the beta cells of the pancreatic islets, leading eventually to irreversible atrophy. Subsequently, permanent diabetes was produced by purified growth hormone (Campbell, Lei and Davidson, 1951). It has also been suggested that the growth hormone stimulates the liberation of insulin from the pancreas of the rat (Milman and Russell, 1950) and of the cat (Milman, DeMoor and Lukens, 1951). It is apparent that interpretation of the causation of the changes now observed in dogs given growth hormone requires consideration of a complex situation wherein the effects upon the tissues of excess amounts of growth hormone may be associated in the early phases with increased liberation, and later with decreased liberation of insulin from the pancreas.

It was concluded by Campbell, Munroe, Hausler and Davidson (1953) that the presence of functional pancreatic islet tissue tends to counteract the deposition of fat in the livers of dogs given growth hormone, and that the great deposition of fat in the livers of depancreatized dogs given growth hormone with the usual dose of insulin is caused by a relative deficiency of insulin. In the intact dogs given growth hormone there was an imperfect but suggestive correlation between the decrease in the insulin content of the pancreas and the fat content of the liver (Tables 2 and 6). The results

indicate therefore that a relative deficiency of insulin may be involved in the deposition of fat in the livers of these dogs given growth hormone.

In uncontrolled diabetes which followed the withdrawal of insulin from depancreatized dogs, marked neutrophilia occurred (Campbell *et al.*, 1953). This suggests the possibility that the neutrophilia produced by growth hormone in these intact dogs may partly be due to relative insufficiency of insulin. However, in intact dogs the neutrophilia occurred early in the course of injections of growth hormone, and the increase in stab cells, which also occurred, was greater than in the depancreatized dogs without insulin. Neutrophilia occurred also in the dog which resisted the hyperglycaemic effects of the growth hormone. The effect of the growth hormone in producing neutrophilia cannot therefore be attributed to hyperglycaemia or to acidosis, nor can it be ascribed entirely to deficiency of insulin. On the other hand, the possibility that insulin may act with growth hormone to produce the early effects on the blood leucocytes should not be neglected.

It has been shown that growth hormone decreases the blood amino acids of normal and hypophysectomized rats (Li, Geschwind and Evans, 1949), and of hypophysectomized, eviscerated rats (De Jongh, Paesi and Van Vieringen, 1950). The reduction of plasma N.P.N. in the dogs given growth hormone for 1 to 4 days, and the indications of reduced excretion of urea, may be compared with these results. However, as the injections were continued, the plasma N.P.N. of the dogs increased to above the initial values. It is possible that this later rise was associated with relative insulin deficiency (Mirsky, 1939). In depancreatized dogs given growth hormone, with the usual dose of insulin, the plasma N.P.N. increased in some cases, and there is evidence of relative insulin deficiency under these conditions (Campbell *et al.*, 1953). However, the accompanying signs of some kidney damage in the diabetic dogs given growth hormone does not permit of interpretation of these changes at present.

In depancreatized dogs without insulin the plasma fibrinogen and the ESR were not changed significantly, so that in dogs given growth hormone the increases in these respects cannot be attributed to relative lack of insulin. Little more can be said at the present time on this subject of pituitary-pancreatic relationships except to point out the possibility that some of these effects of the growth hormone, e.g., the effects on the plasma proteins, may be due to its combined action with insulin.

These effects produced in dogs by the administration of purified growth hormone may be considered in relation to various systems of the body. Since the clotting time of the blood and the prothrombin time of the plasma were decreased, it is evident that the clotting system was activated. The increased fibrinogen concentration of the plasma caused by the growth

hormone probably influences the clotting of the blood. Certain of these effects of the growth hormone may be important in relation to the circulatory system. The effects which may be mentioned in this regard are the increase in blood volume (by increase in plasma volume), reduction in the viscosity of the blood and increase in that of the plasma, increase in ESR and the tendency to sludging which appeared to occur in small vessels. Little can be said regarding the possible effects on the excretory system, but it may be pointed out that after an initial decrease, the plasma N.P.N. increased when the growth hormone was given and that histological changes, to be described later, occurred in the kidneys. Certain "renotropic" effects of growth hormone in dogs have been described by White, Heimbecker and Rolf (1951). Effects of growth hormone on the haematopoietic system are seen in the increased amounts of protein in the plasma and in the increased numbers of neutrophil granulocytes and stab cells that appear in the blood. These responses suggest that the growth hormone may be important in relation to the activity of the reticulo-endothelial system in enhancing immunity to infective agents, as has already been indicated by the studies of Selye (1951) in rats.

SUMMARY

1. The administration of purified growth hormone (3.5 mg./kg./day) to 4 intact fed dogs elicited severe diabetes of such intensity that one animal died after 7 days in diabetic coma. Hyperglycaemia, glycosuria, polyuria, polydipsia, ketonuria and lipaemia were pronounced. During a period of rest these signs disappeared. Reinjection of a smaller dose (2 mg./per/day) again elicited these signs in 2 dogs, but not in the other.

2. The plasma volume was increased in the dogs given growth hormone, thereby increasing the blood volume. The blood cell volumes of the animals (per kg. body weight) were not altered significantly. The volume per cent of red cells, the red cell counts and the haemoglobin concentrations of the blood were decreased, through dilution of the cells with the increased volumes of plasma.

3. The amount of total protein in the blood plasma, per kg. body weight, was increased by the growth hormone injections, due chiefly to the increased volume of plasma. The amounts and concentrations of fibrinogen were greatly increased by growth hormone. The evidence indicates that the rate of production of this protein by the liver was increased in the dogs given growth hormone. Plasma globulin was increased in amount due to the increase in plasma volume and some increase in concentration. The effects of the growth hormone on the amounts of albumin were not definite; while the concentrations were usually decreased, giving low A/G ratios.

4. The ESR was increased markedly by the growth hormone. This effect can be attributed chiefly to the increase in plasma fibrinogen.

5. The clotting time of the blood and the prothrombin time of the plasma were decreased by the administration of the growth hormone.

6. The viscosity of the blood was decreased by the growth hormone, by reduction of the number of cells per unit volume of blood. The viscosity of the plasma was increased, presumably by increase in the concentration of fibrinogen and other large molecules.

7. The insulin extractable from the pancreas was low in the dogs given growth hormone, being 1 to 2% of the average control value in 3 cases, and about 20% of the control value in the most resistant dog.

8. The administration of the growth hormone produced leucocytosis, due to great increase in the number of neutrophil granulocytes and lesser increases in stab cells. The numbers, per unit volume of blood, of lymphocytes, monocytes, eosinophils and basophils, were not much altered by the growth hormone.

9. In the dogs given growth hormone, the lipid and the fat-free solid contents of the liver were greatly increased. The weights of the kidneys were also increased, though to a much less extent.

10. There were indications of "sludging" of blood, as it flowed through the vessels of the mesentery, in the dogs given growth hormone.

Acknowledgments

We wish to thank Professor C. H. Best for his advice, support and encouragement that made this work possible. We are indebted to Dr. G. A. Wrenshall for the determinations of the insulin content of the pancreas, to Mr. D. B. W. Reid, Department of Biometrics, for aid in statistical analysis, to Professor P. H. Greey for bacteriological tests and to Dr. C. C. Lucas for assistance with the manuscript.

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THE METABOLIC PATTERN OF C^{14}-DIETHYLSTILBESTROL[1]

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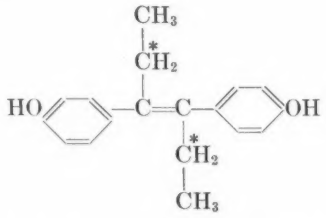
INTRODUCTION

AS IS well established, diethylstilbestrol is a potent synthetic estrogen capable of producing vaginal estrus response in ovariectomized rats in amounts less than one gamma. Although studies on the fate of this compound at rather high dosage levels have been reported (Dodgson *et al.*, 1948; WilderSmith and Williams, 1948; Mazur and Shorr, 1942; Hanahan *et al.*, 1951; Twombly and Schoenwaldt, 1951), no data are available on its metabolism at near physiological levels. The availability of radioactive diethylstilbestrol, labeled in the methylene position with carbon 14 as indicated in the formula,



of sufficiently high specific activity permitted working with doses as low as 5 gamma. A study of this estrogen's metabolic pattern, including its absorption and excretion pathways, was undertaken and the results are reported here.

EXPERIMENTAL

Carbon-14 Diethylstilbestrol

Diethylstilbestrol, labeled with carbon 14 in the methylene position, was synthesized by the method of Dauben and Meikle (unpublished re-

Received for publication February 26, 1953.

[1] This work was performed under Contract AT-(45)-247 between the University of Washington and the Atomic Energy Commission.

sults), which followed essentially the original procedure of Dodds *et al.* (1939). A similar synthetic procedure has been reported by Twombly and Schoenwaldt (1951). The final product had a specific activity of 22.7μc/mg. Its estrogenic activity, as determined by a modified Allen-Doisy test, was the same as that obtained for non-labeled diethylstilbestrol. In some of the experiments the estrogen was administered in dilute ($\sim$0.002 N) alkaline solution; in others, it was given in corn oil. When stored at 4° C., these preparations were shown by spectrophotometric and biological-assay to be stable for at least 30 days.

Assay of Radioactivity

The counting equipment consisted of a Tracerlab Autoscaler and SC-16 Flow Counter. The carbon 14 content of a sample was determined by counting either a direct amount of the material on copper disks or combustion of the sample to CO_2 and conversion to barium carbonate, which was then counted in the usual manner. All samples were counted a sufficient length of time to insure less than a 4% probable error.

Animal Experiments

Adult, female rats of the Sprague-Dawley strain, weighing 200 to 250 gm. were used. In those experiments where expired CO_2 was to be collected the animals were placed in an all glass metabolism cage connected to an alkali absorption tower. Whenever urine contamination of the feces was to be avoided, urethral catheters were used. Bile fistulas were performed in the usual manner. Thoracic duct cannulation was accomplished by the technique described by Bloom *et al.* (1950). Both the bile fistula and lymph fistula animals were maintained in restraining cages and were fed *ad libitum*. The lymph fistula animals were given 1.1% NaCl as drinking water for the first 24 hours, then 0.5% NaCl for the remainder of the experiment. Vaginal estrus response was determined by the Allen-Doisy technique.

RESULTS

Effect of Route of Administration on Distribution of Activity

Adult, female rats were given a single 5 μg. dose of labeled estrogen in alkali by oral or subcutaneous injection. At the end of 6 hours, the animals were sacrificed and the various tissues and organs were removed and analyzed for their C^{14} content. The distribution of radioactivity in these animals is given in Table 1.

As is evident from these data, 49 to 51 % and 60 to 66% of the orally and subcutaneously injected activity, respectively, was found in the G-I tract. No radioactivity was detected in the expired air, indicating that the

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN RATS GIVEN C^{14}-DIETHYLSTILBESTROL 5 μgs., s.a. 25,000 c./m./μg., administered by oral or subcutaneous injection. Six hour experimental period

	Oral			Subcutaneous		
	Recovery, % I.D.			Recovery, % I.D.		
Ovary, adrenals, pituitary, brain, spleen	none	none	none	none	none	none
Kidney	none	1	5	none	1	none
Liver	6	9	6	none	19	2
Blood	none	none	none	none	3	none
Urine	2	none	none	4	none	2
Gastrointestinal tract and contents plus feces	49	54	57	66	63	60
Total recovery	57	64	68	70	86	64

compound was not oxidized to CO_2 in this time period. The only organ in which there was any significant localization of carbon 14 was the liver. On the basis of the results obtained in the bile fistulas experiments described below, it would appear that this most likely represents a temporary storage site of the estrogen before release into the intestinal tracts or represent an average amount of estrogen maintained in the enterophepatic circulatory system (Cantarow *et al.*, 1942, 1943). A similar distribution pattern has been reported in animals given much higher doses (approx. 2 mg.) of this estrogen (Hanahan *et al.*, 1951; Twombly and Schoenwaldt, 1951).

Pathway of Absorption

Inasmuch as diethylstilbestrol is quite different chemically from the natural occurring estrogens and exerts a high estrogenic activity when administered orally, it was of interest to investigate its possible route of absorption from the intestinal tract and the influence of the estrogen solvent, either dilute alkali or oil, on this pathway. The following two techniques were adopted to serve as criteria for the absorption of this labeled estrogen: (a) the presence of radioactivity in the lymph of thoracic duct cannulated, ovariectomized animals and (b) the occurrence of vaginal estrus in the same animals. The amount of estrogen, which was administered to these animals, was that calculated to insure 100% estrus response in non-cannulated, ovariectomized animals (considered to be controls). Thus, if the estrogen were absorbed by two different pathways, i.e. the lymph and the portal systems, then both the radioactivity content of the lymph and the vaginal estrus response should indicate this fact. However, if absorption occurred via the portal system only, then the estrus response should be obtained and no radioactivity should be present in the lymph.

Ovariectomized rats with thoracic duct cannulae were given 10 μgs. of

C^{14}-diethylstilbestrol in alkali or corn oil by intragastric intubation. The lymph was collected at 6 hour intervals up to 48 hours and analyzed for its carbon 14 content. Vaginal smears were taken at 36 and 48 hours and assayed. The results of these experiments are recorded in Table 2.

TABLE 2. ABSORPTION OF C^{14}-DIETHYLSTILBESTROL

10 μgs. C^{14}-diethylstilbestrol, s.a. 25,000 c./m./μg., given orally to a thoracic duct cannulated, ovariectomized rat

Exp. No.	Solvent for estrogen	Counts C^{14} per minute in lymph[a]	Vaginal estrus response at	
			36 hours	48 hours[b]
A-1	Alkali	None	± ± ±	+
A-2	Alkali	None	± ±	± ±
A-3	Alkali	None	+	± ± ±
A-4	Alkali	None	±	±
A-5	Alkali	None	±	± ± ±
O-1	Corn oil	None	±	+
O-2	Corn oil	None	± ±	± ±
O-3	Corn oil	None	+	± ±
O-4	Corn oil	None	± ±	± ± ±

[a] Assayed at 6 hour intervals. In every experiment, over 50 per cent of the injected radioactivity was recovered in the feces.

[b] These responses were interpreted as follows: +, cornified cells only; ± ±, mainly cornified cells, few nucleated epithelial cells, no leucocytes; ± ± ±, predominantly cornified and nucleated epithelial cells, very few leucocytes; ±, leucocytes and nucleated epithelial cells, some cornified cells; −, mainly leucocytes. Typical positive smear is ± ± ±, with + and ± ± considered as positive responses.

As is evident from these data, it is very striking that regardless of the nature of the solvent, in every case a vaginal estrus response was obtained but no radioactivity was detected in the lymph. Although in the corn oil experiment it was visibly evident that the oil was being absorbed via the lymph, no radioactivity was detectable therein. If as little as 1% of the administered dose had been absorbed by the lymph, it would have been detected. It appears from these data that the main pathway of absorption of diethylstilbestrol is not the lymph, but most probably is the portal system.

Release Via the Bile

The evidence presented in the above experiments suggested that a main path of release of the radioactivity from the body was the bile. Therefore, C^{14}-diethylstilbestrol in alkali in amounts ranging from 5 to 100 μgs. was administered by subcutaneous or intravenous injection to adult, female rats with biliary fistulas. The bile was collected at intervals from 1 to 48 hours and analyzed for its carbon 14 content. The results are recorded in Table 3.

These data show that 59 to 87% of the dose administered subcutaneously and 53 to 62% of the amount given intravenously were recovered in the

TABLE 3. RADIOACTIVITY IN BILE OF FEMALE RATS WITH BILIARY FISTULAS GIVEN SUBCUTANEOUS AND/OR INTRAVENOUS INJECTION OF C^{14}-DIETHYLSTILBESTROL

Exp. No.	Injection route	Amount administered		Counts C^{14} per min. in bile in				Recovery*	
		μg.	Counts C^{14} per min.	0–6 hrs.	6–12 hrs.	12–24 hrs.	24–48 hrs.	Counts C^{14} per min.	% of Injected dose
1	Subcutaneous	100	2,500,000	2,000,000				2,000,000	80
2	Subcutaneous	100	2,500,000	2,100,000				2,100,000	84
3	Subcutaneous	20	500,000	435,000				435,000	87
4	Subcutaneous	20	500,000	300,000				300,000	60
5	Subcutaneous	20	500,000	300,000	20,000			320,000	64
6	Subcutaneous	10	250,000	125,000	36,000	25,000	12,500	198,500	79
7	Subcutaneous	5	125,000			79,000†		79,000	63
8	Subcutaneous	5	125,000			73,000†		73,000	59
9	Subcutaneous	5	125,000				98,000‡	98,000	78
10	Subcutaneous	5	125,000				95,000‡	95,000	77
11	Intravenous	10	250,000	133,000				133,000	53
12	Intravenous	10	250,000	156,000				156,000	62

* Less than 5% of the injected dose was excreted in 48 hours in the urine of any of these animals.
† Represents total collection of 24 hours.
‡ Represents total collection of 48 hours.

bile. In none of the experiments was more than 5% of the radioactivity found in the urine. On the basis of these results, it is apparent that the biliary system represents a main metabolic pathway of this synthetic estrogen.

In reference to the latter point, the possibility existed that this synthetic estrogen underwent an enterohepatic circulation similar to that proposed by Cantarow *et al.* (1942, 1943) for the naturally occurring estrogens. In order to consider this theory more fully, the following experiments were performed. Ten μgs. of C^{14}-diethylstilbestrol in alkali were injected subcutaneously into a female rat (A) with a biliary fistula. The bile was collected from this animal for 12 hours and aliquots removed for carbon 14 analysis (Table 4). Immediately thereafter this freshly collected bile was administered by gravity via a plastic cannula to another bile fistula rat (B). The point of entrance of the cannula into Rat B was just above the entrance of the common bile duct into the small intestine and below the cannula insert for bile collection. The rate of administration of the bile

TABLE 4. RECIRCULATION OF BILIARY ESTROGEN

10 μgs. C^{14}-diethylstilbestrol, s.a. 25,000 c./m./μg., given to bile fistula Rat A. Bile collected from A was administered via intestinal cannula to bile fistula Rat B

Exp. No.	Bile from donor rat A	Bile from recipient rat B				
		Counts C^{14}/min. in			Recovery	
	Counts C^{14}/min.	6 hrs.	12 hrs.	24 hrs.	Counts C^{14}/min.	% of injected dose
I	165,200		63,000	37,400	100,400	62
II	97,500	36,900	55,000	4,600	96,500	99
III	123,000	84,700	10,700	3,000	98,400	80
IV	101,100	50,200	30,500	4,100	84,800	84

from Rat A corresponded to the rate of release of bile from Rat B. The bile from the latter animal was collected at various intervals and analyzed for its carbon 14 content. The data obtained with four different animals are given in Table 4.

Sixty-two to 99% of the administe ed radioactivity in the bile from Rat A was reabsorbed by Rat B. It is convincingly evident from these data that the estrogen released in the bile, mainly in the conjugated form (see below), was reabsorbed and thus underwent enterohepatic circulation. The relationship of this phenomena to the maintenance of ovarian activity is being studied.

Of further interest is the fact that several workers (Dodgson *et al.*, 1948; WilderSmith and Williams, 1948; Mazur and Shorr, 1942) have reported that in rabbits given large doses (over 30 μgs.) of diethylstilbestrol, 35 to 45% of the estrogen could be recovered in the urine as the monoglucuronide. However, at rather moderate dosage levels (2 to 3 mgs.), Hanahan *et al.* (1951), using the rat as the experimental animal, and Twombly and Schoenwaldt (1951), using the mouse and rabbit, found that the release into the intestinal tract via the bile represented a main pathway of metabolism of diethylstilbestrol. Our present evidence on the metabolic fate of this estrogen at very low doses shows a similar metabolic pattern. Although the dosage level might govern the excretory pathway, no satisfactory explanation of these apparent anomalies can be given.

Nature of the C^{14} Compounds in Bile

A fractionation of the radioactive compounds present in the bile was undertaken. The bile was collected from each animal for 6 to 8 hours, adjusted to pH 3.5 and extracted continuously with peroxide-free diethyl ether for 24 hours in a liquid-liquid extractor. The either soluble fraction was assayed for its C^{14} content and the results recorded in Table 5. Eighty-one to 88% of the total radioactivity of the bile was ether soluble. In order to determine the chemical nature of the radioactivity in the bile, the ether soluble fraction was subjected to a fractionation procedure similar to that

TABLE 5. FRACTIONATION OF RADIOACTIVITY IN BILE OF FEMALE RATS WITH BILIARY FISTULAS
50 μgs. C^{14}-diethylstilbestrol, s.a. 25,000 c./m./μg., administered subcutaneously in alkali

Fraction	Rat I	Rat II	Rat III	Rat IV
	Counts C^{14}/min.	Counts C^{14}/min.	Counts C^{14}/min.	Counts C^{14}/min.
Original Bile	790,000	570,000	652,000	650,000
Ether Soluble Fraction of Bile (A)	660,000	490,000	575,000	525,000
Ether Soluble, $NaHCO_3$ Insoluble Fraction of A	52,000	70,000	120,000	120,000
$NaHCO_3$ Soluble Fraction of A	590,000	400,000	425,000	395,000

described by Teague and Brown (1951). Over 75% of the C^{14} present in the ether soluble fraction was extractable by 10% $NaHCO_3$, and was considered to be the weakly acidic fraction. Conjugated derivatives of stilbestrol possessing a free carboxyl group, such as the glucuronide, would be soluble to 10% $NaHCO_3$. Upon acidification of this fraction, all the radioactivity was re-extracted into ether. In a typical experiment, 25 mgs. of non-radioactive diethylstilbestrol (m.p. 170.2–170.5°) was added to such an ether soluble fraction which contained 590,000 counts per minute. The ether was removed at 30° under N_2 and the residue suspended and refluxed in 1 N H PO_4 for 1 hour. The hydrolysate was extracted with diethyl ether, washed 4 times with equal volumes of water and the ether solvent removed by evaporation under N_2. The residue was dissolved in 95% EtOH and the stilbestrol was crystallized by the addition of water. It was then recrystallized from aqueous ethanol until the melting point and specific activity were constant. Usually this required three recrystallizations. Two additional recrystallizations from ether-benzene did not alter the melting point or the specific activity. Fifteen milligrams of radioactive diethylstilbestrol (m.p. 170.3–170.7°), specific activity 23 counts per microgram, were obtained. Calculation by the usual isotope dilution equation showed that 23 μgs. of diethylstilbestrol were present in the weakly acidic fraction; theoretical yield from the amount of radioactivity originally present was 24 μgs. This indicated that 96 % of the administered estrogen was present in this fraction, possibly in the form of a monoglucuronide.

Discussion

The evidence obtained on the recirculation of diethylstilbestrol as a conjugate, possibly the glucuronide, in the bile lends considerable support to an enterohepatic circulation of estrogens similar to that suggested by Cantarow *et al.* (1942, 1943). As suggested by Fishman (1951), estrogens might act biologically as the glucuronide. However, in reference to the action of diethylstilbestrol this does not seem too probable inasmuch as conjugates of this compound, in particular the glucuronide, have weak estrogenic activity when administered by the oral or subcutaneous route. It would seem more likely that the diethylstilbestrol conjugate represents a pooled storage form of the estrogen from which the free estrogen can be liberated enzymatically in small amounts as required for the maintenance of ovarian activity and other necessary functions. Preliminary evidence has shown that all of the diethylstilbestrol in the "recirculated" bile is in the conjugated fraction. Thus, as suggested above, this could represent a storage form of this estrogen. Moreover, it was shown that rats with ure-

thral fistulas excreted the major portion of the administered radioactivity in the feces (less than 3% appeared in the urine).

The results on the pathway of absorption of diethylstilbestrol are interesting. It is evident from these data that this estrogen is absorbed by a path other than the lymph system, most probably the portal system. Inasmuch as the nature of the solvent had no effect on the absorption route, it would appear from our results that the incorporation of this estrogen into the portal system, and then to the biliary system represents a primary reaction in the establishment of a pool of available estrogen.

SUMMARY

The metabolic pattern of radioactive diethylstilbestrol, labeled with carbon 14 in the methylene position, in the adult, female rat has been investigated. Release of a conjugated derivative of this labeled estrogen via the bile represents a major path of metabolism. Evidence supporting the theory of enterohepatic circulation of estrogens has been obtained. Regardless of the solvent in which the estrogen is administered orally, absorption occurs by a route other than the lymph system, most probably the portal system.

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THE EFFECT OF CORTISONE ON THE THYROIDAL AND RENAL METABOLISM OF IODINE

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AMONG the many physiological alterations which follow the administration of ACTH or cortisone, apparent changes in the thyroidal metabolism of iodine have received recent attention. Both in animals and in man, these agents have been found to reduce the thyroidal accumulation of a tracer dose of radioactive iodine. (Wolfson *et al.*, 1950; Hill *et al.*, 1950; Frederickson *et al.*, 1952; Kuhl and Ziff, 1952; Money *et al.*, 1950, 1951). Similar alterations have been noted in animals receiving stressor agents (Soffer *et al.*, 1949; Paschkis *et al.*, 1950; Money *et al.*, 1950; Williams *et al.*, 1951). Since the abnormalities in the thyroidal radioactive iodine accumulation resulting from the administration of cortisone can be at least partially reversed by the administration of pituitary thyrotropin (TSH), it has been suggested that the changes produced by cortisone are the result of a diminution in the secretion of TSH by the pituitary gland (Hill *et al.*, 1950). More recently, however, it has been demonstrated that, in hypophysectomized rats, cortisone reduces the response to administered TSH, as measured by the thyroidal content of radioactive iodine 24 hours after the administration of a tracer dose of this isotope (Woodbury *et al.*, 1951; Paschkis *et al.*, 1952). From these observations it was concluded that cortisone may exert a depressant action directly on the thyroid gland, diminishing its response to TSH. The present communication reports the results of experiments designed to elucidate the nature of the effect of cortisone upon the accumulation of radioactive iodine by the thyroid glands of hypophysectomized rats. The observations herein presented do not necessarily bear upon the complex situation which may result when cortisone is administered to the intact or to the adrenalectomized animal.

The observed effect of cortisone on the thyroidal I^{131} content of hypophysectomized rats may be explained in several ways. Since it has been clearly demonstrated that cortisone does not accelerate the rate of release of radioactive hormone from the thyroid gland, this possible mechanism

Received for publication February 26.

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was not investigated further (Perry, 1951; Albert, Tenney and Ford, 1952). Cortisone may reduce the capacity of the thyroid gland to remove iodide from the serum (thyroidal clearance of plasma iodide), or it may so alter the extrathyroidal metabolism of iodide that, over a given time interval, less radioactive iodide is available to the gland for accumulation. This could be accomplished by means of an increase in tissue avidity for iodide ion, an increase in the rate of renal excretion of iodide, or a combination of these mechanisms. These possibilities were evaluated in the present studies. In hypophysectomized rats, either receiving or deprived of exogenous TSH, measurements were made of the influence of cortisone on both urinary and thyroidal clearances of iodide and on the rate of disappearance of injected radioactive iodide from the plasma. A more rapid loss of injected I^{131} from the plasma of animals treated with cortisone was observed. This increased rate of loss was sufficient to account for a decreased thyroidal accumulation of I^{131}, and could be explained by an increase in the renal clearance of iodide which occurred in animals given cortisone.

METHODS

All rats used in this study were received from a commercial source[2] ten days following hypophysectomy and were albino males. All animals weighed 80 to 100 gm. at the time of operation. Proof of completeness of the hypophysectomy depended upon the demonstration of weight loss, gonadal and adrenal atrophy. All animals were maintained on isotonic saline as their source of fluid. In animals in whom the formation of thyroid hormone was allowed to proceed, the diet consisted of fresh horse meat, bread and oranges. In those animals in which the protein-binding of iodine was to be inhibited, the diet consisted of ground Purina checkers containing 0.1% propylthiouracil. In all instances in the latter groups, propylthiouracil was administered for at least four days prior to the first injection of either TSH or cortisone. TSH[3] and cortisone[4] were administered in five equally divided doses at intervals of 12 hours, the last dose being given 12 hours prior to the administration of I^{131}. Radioactive iodine (2–5 μc.) in the carrier-free state was administered in 0.5 cc. of isotonic saline solution, delivered intraperitoneally from a tuberculin syringe. The animals were so selected that within each treatment-group, the mean weights of the time-subgroups were equal. Three separate experiments were carried out. At the time of each experiment, the animals were 20–21 days post-operative.

Experiment I

Thirty-five rats were divided into six groups. All animals received propylthiouracil. In groups A, B and C, each animal received a total dose of 1.0 mg. of TSH. Groups D, E and F received no TSH. In groups A and D, animals received a total dose of 12.5 mg. of cortisone. In groups B and E, a total of 6.25 mg. of cortisone was administered. Groups C and F received no cortisone. Animals were killed 60–90 minutes after the administration of I^{131}.

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[2] Hormone Assay Laboratories, Chicago, Illinois.

[3] Thyrotropin, Armour, Lot K46808R, kindly supplied by Dr. C. J. O'Donovan of Armour Laboratories. Dosages cited in mg. equivalent to Armour standard (2R3). Potency approximately 0.5 U.S.P. μ/mg. equivalent.

[4] Cortone Acetate, Merck.

Experiment II

Propylthiouracil was not administered. Sixty-four animals were divided into four groups. In groups A or B, each animal received a total dosage of 1.5 mg. of TSH. Groups C and D received no TSH. A total dose of 20 mg. of cortisone was administered to each animal in groups A or C. Groups B and D received no cortisone. Each group was further subdivided into four time-subgroups which were killed, respectively, at 2, 4, 6 and 8 hours following the administration of radioactive iodine.

Experiment III

All animals received propylthiouracil. Eighty animals were divided into four groups. Each animal in group A or B received a total dosage of 1.0 mg. of TSH. Groups C and D received none. Groups A and C received a total of 25 mg. of cortisone. Groups B and D received none. Each group was further subdivided into four time-subgroups to be killed at 2, 4, 6 and 8 hours, respectively, following the administration of radioactive iodine. In addition, four animals from each treatment-group were placed in metabolism cages and collections of urine were made at intervals of two hours for the first eight hours following the administration of radioactive iodine.

In all instances, hormones were administered in five equal doses, given at intervals of 12 hours, and ending 12 hours prior to the injection of I^{131}.

In experiment I, blood was drawn from the hearts of unanesthetized animals which were then killed immediately by a blow on the head. In subsequent experiments blood was drawn from the abdominal aorta after the animal had been fully anesthetized with secobarbital.[5] In no instance did more than two minutes elapse between the completion of the withdrawal of blood and the completion of the thyroidectomy.

In experiment I, samples of plasma and thyroidal fractions were studied individually. In experiments II and III, thyroid glands and plasma samples from animals in each time-subgroup were pooled. In experiment II, plasma I^{131} was fractionated into inorganic and protein-bound moieties by trichloroacetic acid separation. The inorganic fraction was assayed for radioactivity. In experiments I and III, the radioactivity of the plasma was measured without preliminary fractionation.

Thyroid glands were homogenized in ground glass homogenizers containing 10% trichloroacetic acid. Subsequent washings of the precipitate were carried out with 2.5% trichloroacetic acid and with distilled water. The combined supernatant solutions were considered to contain thyroidal inorganic iodide. The remaining acid-insoluble residues, containing hormonal or protein-bound I^{131}, were digested in 2N NaOH. Determinations of radioactivity were carried out in the conventional fashion, using a mica-window Geiger-Muller tube. The radioactivity of each sample was compared with that of an appropriately diluted sample of the administered dose. Appropriate corrections for self-absorption were made in the observed radioactivity of each sample.

The following mathematical considerations were employed:

Wt = mean thyroid weight (mg.)
t = time after administration of I^{131} (hours)
P = plasma I^{131} concentration at time, t (% dose/cc.)
P_0 = plasma I^{131} concentration when $t = 0$
$100/P_0$ = iodine distribution space (cc.)
A = amount of I^{131} collected in thyroid or urine up to time, t (% dose/gm. thyroid or % dose excreted in urine)
k = rate constant of disappearance of iodide from the plasma (proportion/hour)

[5] Seconal, Squibb.

The thyroid/serum iodide concentration ratio was calculated from the formula

$$\text{ratio} = A \text{ (inorganic)}/P \tag{1}$$

The method of calculation thyroidal and renal clearances was similar to that described by Berkson *et al.* (1950). For each treatment group, the exponential curve of decline, with time, of the plasma I^{131} concentration was plotted by the "least squares" method (Snedecor, 1946) yielding for each group an equation of the type,

$$\log P = \log P_0 + mt \tag{2}$$

$$k = -2.303\ m, \text{ where } P = P_0 e^{-kt}$$

$$\frac{dA}{dt} = CP = CP_0 e^{-kt}$$

Since when

$$t = 0, \quad A = 0,$$

$$A = \frac{CP_0}{k}(1 - e^{-kt}) \tag{3}$$

From equation (3), the ultimate amount of I^{131} collected $(t = \infty) = CP_0/k$ (4)
In the calculation of urinary clearances between two time periods, t_1 and t_2,

$$C = (A_2 - A_1)k/P_0(e^{-kt_2} - e^{kt_1}) \tag{5}$$

The thyroidal clearances were calculated for each time-subgroup for the entire interval from $t = 0$ until the group was killed. Renal clearances were calculated for each individual collection period. The mean thyroidal or renal clearance is the mean of the individual values so obtained. The mean clearance values were used with equation (3) to calculate the 24 hour collection, and with equation (4) to calculate the ultimate collection of I^{131} in either thyroid or urine.

Total plasma iodide clearance $= 100\ k/P_0$
Extrathyroidal clearance =

$$\text{plasma clearance} - \frac{\text{thyroidal clearance} \times wt.}{1000}$$

Tests for the statistical significance of the differences between measurements from the various groups were carried out by the "*t*-test" of Fisher (Snedecor, 1946). All changes judged to be significant had a *p*-value of less than .01.

RESULTS

In no experiment did cortisone significantly influence the ratio of thyroidal mass to body weight, regardless of whether the animals had or had not received TSH. In experiments I and III, in which propylthiouracil was administered, the thyroidal content of organically-bound I^{131} was negligible in all groups.

Experiment I (Table 1): The mean thyroid/plasma iodide concentration

TABLE 1. THE COMBINED EFFECTS OF CORTISONE AND TSH ON THE THYROID/SERUM IODIDE RATIO IN HYPOPHYSECTOMIZED RATS FED A DIET CONTAINING 0.1% PROPYLTHIOURACIL*

Group	A (6 rats)	B (6 rats)	C (6 rats)	D (6 rats)	E (5 rats)	F (6 rats)
TSH (mg.)	1.0	1.0	1.0	0	0	0
Cortisone (mg.)	12.5	6.25	0	12.5	6.25	0
Thyroid weight (mg.)	6.2 (5.0–7.4)	7.5 (6.3–8.7)	8.0 (6.0–9.9)	6.0 (4.9–7.2)	6.5 (4.2–8.8)	6.3 (4.8–7.4)
Plasma I^{131} (% Dose/cc.)	1.14 (0.75–1.49)	1.56 (1.19–2.00)	1.22 (0.93–1.48)	1.17 (0.95–1.46)	1.34 (1.18–1.57)	1.50 (1.20–1.73)
Thyroid/plasma Iodide ratio†	143 (121–160)	150 (110–165)	142 (125–164)	6.2 (4.2–8.8)	12.3 (6.6–14.2)	8.8 (6.4–10.5)

* Values given for each group represent mean and range for each group.
† I^{131}-iodide/gm. thyroid/I^{131}/cc. plasma.

ratios in groups not given TSH varied from 6.2 to 12.3. In animals given TSH, the mean ratios in the three treatment-groups varied from 142 to 150. In neither those animals which were treated with TSH, nor in those animals which were not, did cortisone significantly affect the concentration ratio.

Experiment II (Tables 2 and 3): The rate at which I^{131} disappeared from the plasma of animals which received cortisone markedly exceeded the disappearance rate found in animals not so treated, and was further augmented by the administration of TSH. The calculated values for the total plasma iodide clearance were altered in a similar direction.

Calculation of thyroidal clearances of plasma iodide, however, revealed that changes induced by cortisone in the total rate of clearance of plasma iodide could not be explained by changes in thyroidal function. Neither in the group of animals which received TSH, nor in the group which did not, was the mean thyroidal iodide clearance significantly affected by cortisone. Nevertheless, calculation of either the 24-hour or the ultimate thyroidal uptake of radioactive iodine revealed that in both of these groups cortisone markedly decreased this parameter of thyroid function. Thus, a predicted ultimate uptake of 110.8%/gm. of thyroid weight, calculated for those animals receiving neither TSH nor cortisone, was decreased to 53.5%/gm. in those animals which received cortisone. In those animals which received TSH, the comparable values were 1006% and 627%/gm. of thyroid weight, respectively. In all four groups of animals the clearance values obtained at the two-hour period were considerably higher than those obtained in the subsequent periods.

Experiment III (Tables 2 and 3): In this experiment, the rate of disappearance of iodide from the plasma was again found to be markedly increased by the administration of cortisone. The disappearance rate was 5.7%/hour in those animals receiving no treatment and 5.2%/hour in those animals receiving TSH alone. In contrast, the disappearance rate

TABLE 2. THE INFLUENCE OF CORTISONE AND OF TSH ON THE THYROIDAL AND RENAL REMOVAL OF I^{131} FROM THE PLASMA OF HYPOPHYSECTOMIZED RATS[1]

Group	Plasma I^{131} concentration (% dose/cc.)				Thyroidal protein-bound I^{131} (% dose/gm. thyroid)				Thyroidal clearance of plasma iodide (cc./gm./hr.)				Cumulative urinary excretion of I^{131} (% dose excreted)				Urinary clearance of I^{131} (cc./hr.)			
Hours after I^{131}	2	4	6	8	2	4	6	8	2	4	6	8	2	4	6	8	0–2	2–4	4–6	6–8
TSH—1.5 mg. Cortisone—20 mg.	.652	.396	.194	.136	373	344	431	528	206	124	129	143	—	—	—	—	—	—	—	—
TSH—1.5 mg.	.730	.508	.412	.326	351	383	427	575	207	131	110	126	—	—	—	—	—	—	—	—
Cortisone—20 mg.	.740	.422	.248	.172	29.0	31.6	32.7	39.8	15.1	10.4	8.8	9.6	—	—	—	—	—	—	—	—
No therapy	.632	.536	.490	.376	22.0	37.1	36.3	43.7	15.0	14.0	9.9	9.7	—	—	—	—	—	—	—	—
TSH—1.0 mg. Cortisone—25 mg. PTU[2]	.732	.469	.365	.308	—	—	—	—	—	—	—	—	14.4	36.8	51.3	63.6	9.0	18.7	16.0	18.1
TSH—1.0 mg. PTU	1.052	.891	.898	.743	—	—	—	—	—	—	—	—	12.1	19.4	23.8	31.6	6.0	3.4	2.5	4.9
Cortisone—25 mg. PTU	.698	.504	.281	.219	—	—	—	—	—	—	—	—	36.9	58.0	70.1	79.1	21.0	18.0	15.4	16.9
PTU	1.035	.913	.800	.754	—	—	—	—	—	—	—	—	9.7	20.7	30.5	37.4	4.5	5.7	5.7	4.5

[1] Sixteen animals in each treatment group; figures cited are values of pooled specimens from four animals in each of four time-subgroups.
[2] PTU = Propylthiouracil, 0.1% in diet.

TABLE 3. THE INFLUENCE OF CORTISONE AND OF TSH ON THE THYROIDAL AND RENAL REMOVAL OF I^{131} FROM THE PLASMA OF HYPOPHYSECTOMIZED RATS*

Group	Plasma iodide disappearance rate (%/hr.)	Iodide distribution space (cc.)	Thyroidal plasma iodide clearance (cc./gm./hr.)	Ultimate thyroidal I^{131} uptake (%/gm.)	Thyroid weight (mg.)	Urinary iodide clearance (cc./hr.)	Ultimate urinary I^{131} excretion (% dose)
TSH—1.5 mg. Cortisone—20 mg.	27.0	88.6	150 (124–206)	627	6.4±0.3†	—	—
TSH—1.5 mg.	13.2	108.5	144 (110–207)	1006	6.5±0.4	—	—
Cortisone—20 mg.	24.2	85.7	11.0 (8.8–15.1)	53.5	5.2±0.2	—	—
No therapy	8.4	131.0	12.2 (9.7–15.0)	110.8	6.0±0.2	—	—
TSH—1.0 mg. Cortisone—25 mg. PTU	14.1	109.8	—	—	5.8±0.4	15.5 (9.0–18.1)	100.0
TSH—1.0 mg. PTU	5.2	86.4	—	—	5.2±0.4	4.2 (2.5–6.0)	93.5
Cortisone—25 mg. PTU	20.3	93.4	—	—	5.2±0.3	17.8 (15.4–21.0)	94.2
PTU	5.7	87.0	—	—	5.3±0.3	5.1 (4.5–5.7)	102.8

* Values cited represent mean and range of values from the four time-subgroups within each treatment group.
† Standard error of mean.

was found to be 20.3%/hour in animals receiving cortisone alone and 14.1%/hour in animals receiving TSH in addition to cortisone. The more rapid disappearance of radioactive iodine from the blood of animals treated with cortisone could be explained by the significant increase in the renal clearance of iodide which occurred in these animals. The mean renal clearance of iodide was 5.1 cc./hour in those animals which received no treatment and 4.2 cc./hour in those animals receiving TSH alone. In the animals receiving cortisone, the mean clearance was 17.8 cc./hour in those animals receiving no other therapy, and 15.5 cc./hour in those receiving TSH in addition to cortisone. The differences noted between animals given TSH and those not so treated were not statistically significant. Although actual thyroidal clearances of plasma iodide were not measured in this experiment, analyses of the thyroids in the various groups revealed essentially no collection of organically-bound radioactive iodine. It may therefore be concluded that thyroidal clearance of plasma iodine in all four groups was virtually absent. In all groups the total plasma clearance was virtually identical with the calculated mean renal clearance. It would therefore be expected that the calculated values for the ultimate renal excretion of radioactive iodide in all four groups should approach 100%. This proved to be the case. These values were found to be 94.2% and 93.5% in those animals which did not receive cortisone and 102.8% and 100% in those animals which did.

DISCUSSION

The thyroid/plasma iodide concentration ratio in hypophysectomized rats varied between 6.2 and 12.3. These values are in essential agreement with those previously reported by Greer (1949). In accordance with previous observations, the ratio was markedly increased by the administration of TSH (Ingbar and Roberts, 1952; Halmi *et al.*, 1953). In neither instance, however, was there significant evidence of an effect of cortisone on this concentration ratio.

It has recently been pointed out that the thyroidal clearance of iodide, being free from variation caused by differences in the volume of distribution of iodide ion and in its rate of disappearance from the plasma, may be better index of thyroidal function than the accumulation of radioactive iodine within the thyroid gland (Myant *et al.*, 1950; Berson *et al.*, 1952). The present experiments serve to illustrate this point. Although the calculated values for the 24-hour and the ultimate uptake of radioactive iodine by the thyroid glands of hypophysectomized animals were reduced in those animals given cortisone, as compared to those not so treated, the actual clearance by the thyroid of iodide from the plasma was found to be unaltered by the administration of cortisone. It seems likely that the net thyroidal clearance of plasma iodide is dependent upon the magnitude of the concentration ratio existing between thyroidal and plasma iodide, and upon the rapidity of binding of thyroidal iodide to protein. Therefore, failure of cortisone to alter either the net thyroidal clearance of plasma iodide, or the iodide concentration ratio, would indicate that cortisone had also failed to affect the rate of binding of thyroidal iodide to protein. If the failure of cortisone to influence the thyroid/plasma iodide concentration ratio in rats treated with propylthiouracil indicates that cortisone also fails to affect this ratio in rats which are forming hormone, it may be concluded that cortisone did not alter the rate of protein-binding.

The extrathyroidal clearance of iodide was found to be greatly increased in those animals which received cortisone, regardless of whether they had also received TSH or propylthiouracil. Since the kidney is the principal extrathyroidal site of the removal of iodide from the plasma, it appeared likely that renal removal of iodide was accelerated by the administration of cortisone. This proved to be the case. Upon actual measurement, the renal clearance of plasma iodide was increased approximately threefold by the administration of cortisone to hypophysectomized animals regardless of whether or not they had comcomitantly received TSH. By carrying out experiment III in animals treated with propylthiouracil, it was possible to eliminate, almost completely, the thyroidal removal of plasma radioactive iodide as a source of loss of this material from the blood. As might be expected from the fact that the kidney was essentially the sole source

of removal of iodide from the plasma, the calculated values for the ultimate excretion of the administered dose of radioactive iodide in all four groups approached very close to 100%. The agreement, noted in all four groups, between the renal clearance of plasma iodide and the total clearance of plasma iodide suggests that tissue avidity for iodide ion was not significantly affected by the administration of cortisone.

Little is known of the factors, hormonal or otherwise, which regulate the renal excretion of iodide (Riggs, 1952). The present experiments demonstrate that in the hypophysectomized rat, cortisone may decrease the thyroidal accumulation of radioactive iodine by virtue of increasing the renal clearance of this electrolyte. Berson and Yalow (1952) have demonstrated a transient increase in the renal clearance of iodide in humans given ACTH or cortisone. The mechanism by which cortisone brings about an increase in the renal clearance of iodide is uncertain, but the phenomenon is reminiscent of other effects of cortisone on the function of the kidney (Ingbar *et al.*, 1950, 1951; Earle *et al.*, 1951). Boss *et al.*, (1952) have noted a progressive reduction in the glomerular filtration rate of hypophysectomized rats, beginning a few days after operation. It is unlikely, however, that cortisone increases iodide clearance solely by augmenting glomerular filtration rate. Preliminary results in this laboratory indicate that the clearance of endogenous creatinine is not materially affected by the administration of cortisone to rats hypophysectomized 20 days earlier. In addition, Boss *et al.* (1952) have demonstrated little, if any, increase in the clearance of exogenous creatinine following the administration of adrenal cortical extract to rats in which a similar period had elapsed since hypophysectomy.

Albert, Tenney, and Lorenz (1952) have demonstrated that hypophysectomy, unlike adrenalectomy, results in marked diminution in the renal clearance of iodide in the rat. Preliminary observations in this laboratory confirm the failure of adrenalectomy to reduce the renal clearance of iodide, and suggest that this results from the absence, in the adrenalectomized rat, of both 11-desoxy and 11,17-oxygenated steroids. Desoxycorticosterone acetate has been found to reduce the renal clearance of iodide in adrenalectomized rats. This reduction is prevented by the concomitant administration of cortisone. It is difficult, however, to reconcile these data with the report of Migeon *et al.* (1952), that cortisone, when administered to adrenalectomized rats maintained with desoxycorticosterone, produces a delay in the renal excretion of iodide.

Riggs (1949) has reported that, in the dog, the renal clearance of iodide is markedly dependent upon the simultaneous excretion of chloride. It is interesting to note, therefore, that the clearance values obtained in the present study are seven to eight times as great as those reported by Albert *et al.* (1952). The hypophysectomized rats studied by these authors, unlike those

herein presented, were apparently not given supplementary saline.

The data of Woodbury *et al.* (1951) reveal little, if any, effect of ACTH or cortisone on the 24-hour uptake of radioactive iodine in hypophysectomized rats not given TSH. An increased renal clearance of iodide, induced by cortisone, should, however, decrease the late thyroidal accumulation of radioactive iodine, even when TSH is not administered.

The values of the iodide distribution space herein presented have been obtained by extrapolation to zero-time of the curve of plasma radioactivity obtained during the period from two to eight hours after the administration of the tracer. The values thus obtained approximate the body weight of the animals used. There have been few reports of the volume of distribution of radioactive iodide in rats. Calculation of data obtained from approximately 400 other rats studied in this laboratory and from the charts of several other authors (Morton *et al.*, 1942; Taurog *et al.*, 1947; Stevens *et al.*, 1949) reveal distribution spaces which constitute at least 60 to 80 per cent of the body weight of the rat. It has been reported (Jones, 1951) that the volume of distribution of radioiodide in the rat is approximately 30 per cent of the body weight. Possibly, however, these measurements were based on concentrations of radioiodide in plasma obtained early in the course of mixing of this tracer (Albert, Tenney, and Lorenz, 1952; Brownell, 1951).

SUMMARY

Neither the basal level of the thyroid/plasma iodide concentration ratio found in hypophysectomized rats, nor the elevated level found in similar rats treated with TSH was affected by the administration of cortisone. The thyroidal clearance of plasma iodide in hypophysectomized rats was markedly increased by the administration of TSH, but was unaffected by the concomitant administration of cortisone, The rate of disappearance of radioactive iodide from the plasma of hypophysectomized animals was markedly augmented by the administration of cortisone, regardless of whether or not TSH was also given. The rapid decline in the concentration of radioactive iodide in the plasma, noted in animals treated with cortisone, could be entirely explained by an increase in the renal clearance of iodide which occurred following the administration of this hormone, and was sufficient to explain a decrease in the total accumulation of I^{131} by the thyroid glands of animals so treated.

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A STUDY OF THE INTERRELATIONSHIPS OF MATERNAL AND FETAL ADRENAL GLANDS OF RATS[1,2]

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INTRODUCTION

DURING pregnancy in the human (Venning, 1946; Tobian, 1949; Devis and Devis-Vanden Eeckhoudt, 1949; Jailer and Knowlton, 1950; Lloyd *et al.*, 1952) or in the experimental animals, (Anderson and Sperry, 1937) secretory activity of the adrenal cortex of the mother appears to be high. Little is known about the activity of the fetal adrenal cortex and the interrelationships of maternal and fetal adrenals have not been widely studied. Firor and Grollman (1933) observed that adrenalectomized pregnant albino rats survived twice as long as nonpregnant controls. Ingle and Fisher (1938) found an increase in weight of the fetal adrenals if the maternal adrenals had been removed during the pregnancy. Adrenalectomized pregnant rats delivered their litters normally, but did not lactate sufficiently to raise them unless treated with cortical hormones (Tobin, 1939).

Walaas and Walaas (1944) confirmed the findings of Ingle and Fisher and showed that fetal adrenal weight increased 33% if maternal adrenals were removed on the 16th day of gestation. They also reported that the mitotic activity of hypertrophied fetal glands was three times normal and concluded that the increased weight was not only fluid but also new tissue laid down by the glands in response to a lowered titer of maternal cortical hormones. The administration of desoxycorticosterone to the adrenalectomized rats resulted in a depression of the fetal adrenal mitotic activity and weight to the normal range.

Jailer (1950) has studied the pituitary-adrenal axis of the newborn rat and has found that epinephrine or cold causes no change in the concentra-

Received for publication, March 2, 1953.

[1] The work described herein was submitted in partial fulfillment of the requirements for the degree of Masters of Arts in Zoology in the Graduate School of Syracuse University by J. M. Jones.

[2] The ACTH used in these studies was Armour Lot #128-105-R and was supplied through the courtesy of Dr. Edward Hayes and Mr. Irby Bunding of the Armour Company. One mg. of this material has a potency of 1.6 units of La-1-A.

tion of adrenal ascorbic acid until the eighth day of life, but that ACTH can partially deplete the glands of four and six day old rats. He concludes that the adrenal glands of newborn rats have the capacity to respond to pituitary stimulation but that the pituitary cannot respond to epinephrine or cold until at least eight days of age.

Wells, (1948) however, has presented some evidence suggesting the possible elaboration of ACTH by the fetal pituitary. Jost (1952) also has presented data indicating that the fetal pituitary forms and releases ACTH.

The present study was undertaken to answer the following questions:

1. Is the adrenal cortex of the pregnant animal normally responsive to stress and ACTH as measured by changes in cholesterol and ascorbic acid concentration?
2. What is the effect of stimulation of the maternal gland, by stress or ACTH, on the fetal adrenal?
3. What is the effect on fetal adrenal function of exogenous cortisone given to the pregnant animal?
4. Can evidence be obtained concerning the passage of ACTH through the placental barrier?

MATERIALS AND METHODS

Sprague-Dawley rats were used in all of the experiments. They were fed a diet of Purina fox chow and occasional green vegetables.

The pregnant rats weighed from 260 to 350 gm. Nonpregnant female rats, weighing from 170 to 270 gm., which was approximately the weight of the pregnant animals before conception, and pregnant animals were paired according to body weight at the onset of pregnancy. The pregnant rats were sacrificed on the 21st day of the pregnancy.

The animals were sacrificed by a blow on the head. After removal of the maternal adrenals, the uterus was opened and the fetuses removed. The pooled fetal body weight was obtained at this time and the fetuses sacrificed by decapitation and both adrenals were removed.

When both adrenal ascorbic acid and cholesterol concentrations were to be determined for the same litter, one gland was taken from each fetus and the glands were pooled for each of the analyses.

One of the maternal glands was used for each determination. Cholesterol was determined by a modification of the Schoenheimer-Sperry method (Sperry, 1945) and ascorbic acid by the method of Roe and Kuether (1943).

In the experiments to determine the effects of exposure to cold, the pregnant and nonpregnant rats were exposed to a temperature of 4–6° C. for twelve hours. Food and water were available during the period of exposure.

In the experiments to determine the effects of exogenous ACTH, the hormone was given intravenously in three divided doses at hourly intervals through the epigastric or femoral veins to animals maintained under sodium amytal anesthesia. The animals were sacrificed one hour after the last injection.

Pregnant and nonpregnant rats were given a total of 30 mg. of Cortisone Acetate in two or three subcutaneous injections over a fifteen hour period. They were sacrificed three hours after the last injection.

RESULTS

Effect of Exposure to Cold

When nonpregnant rats were subjected to cold for twelve hours, adrenal cholesterol decreased 29%, from a control level of 4.44 mg. to 3.18 mg./100. mg. of adrenal tissue.

Pregnant rats had much lower prestress concentrations of cholesterol than the nonpregnant animals, averaging 2.57 mg./100 mg. of adrenal tissue. Following exposure to cold, the adrenals of the pregnant animals contained an average of 1.10 mg. of cholesterol per 100 mg.

The fetal glands normally contain about 0.80 mg. of cholesterol per 100 mg. of tissue. When the mother was subjected to cold, the fetal adrenal

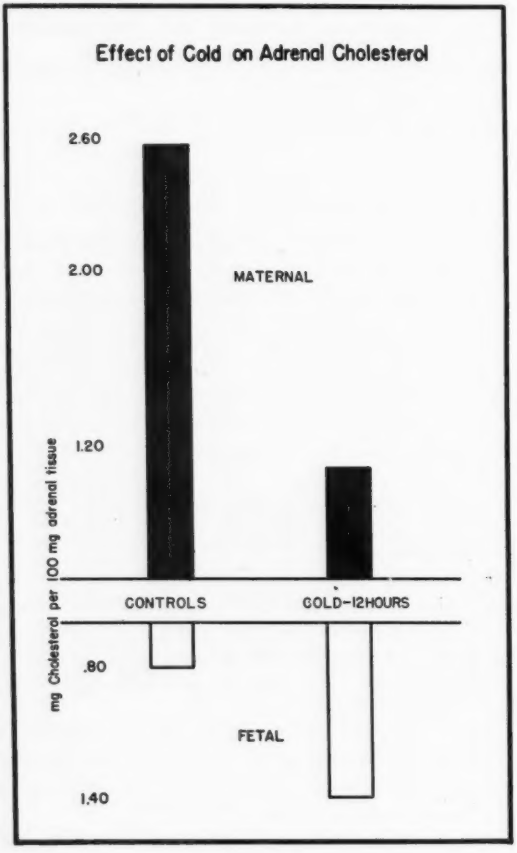

FIG. 1

TABLE 1. THE EFFECT OF EXPOSURE TO COLD ON MATERNAL AND FETAL ADRENAL CHOLESTEROL

	No. of animals	Adrenal cholesterol mg./100 mg.	Per cent change	T value	Significance
Nonpregnant control*	20	4.44			
Nonpregnant stress	18	3.18	28.5	4.16	+
Pregnant maternal control*	14	2.57			
Maternal stress	11	1.10	57	5.97	+
Fetal control	12	0.80			
Fetal stress	11	1.39	75	5.27	+

* The adrenal cholesterol concentration of the pregnant controls is significantly lower than that of the nonpregnant controls.

cholesterol increased to a level of 1.39 mg./100 mg., an increase of 75% See Figure 1, and Table 1.

The changes in adrenal cholesterol are summarized in Table 1.

Effect of Intravenous ACTH—Nonpregnant Rats (Fig. 2)

In the nonpregnant rat, intravenous administration of .008 I.U. of ACTH did not cause significant depletion of adrenal cholesterol and ascorbic acid. A dose of .04 I.U. of ACTH caused only a slight further decrease in adrenal cholesterol, but brought about a significant depletion of ascorbic acid. Very little further depletion of ascorbic acid occurred on increasing the dose of ACTH to 1.6 I.U.

0.16 of a unit of ACTH was no more effective than .04 I.U.; both doses caused a depression of the adrenal cholesterol of 15%. Increasing the ACTH dose to .8 I.U. caused a significant decrease of 28%. When 1.6 I.U. was given, the adrenal cholesterol was 35% lower than that of the normal controls.

It should be noted that although the ascorbic acid level was significantly depressed by a .04 I.U. dose of ACTH, a similar significant depletion of cholesterol did not occur until .8 I.U. of ACTH, i.e., twenty times as much, had been given.

Effect of Intravenous ACTH—Pregnant Rats (Figs. 3 and 4, Table 2)

The adrenal glands of pregnant rats had a much lower concentration of cholesterol than the glands of nonpregnant animals, but the ascorbic acid levels were similar. Injections of either .16 I.U. or 1.6 I.U. of ACTH elicited maternal adrenal responses of equal intensity, causing a 25% decrease of cholesterol and a 50% decrease of ascorbic acid.

The fetal cholesterol increased by 58% while the ascorbic acid decreased by a small, statistically insignificant, amount when the pregnant rats were given .16 I.U. of ACTH.

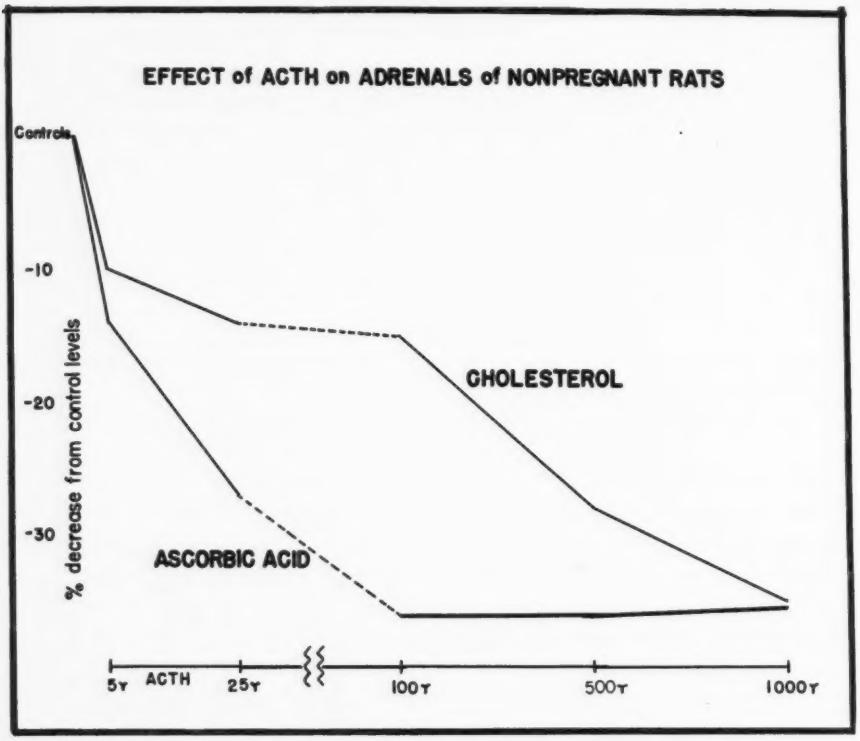

FIG. 2

Following the administration of 1.6 units of ACTH to the mothers, the fetal adrenal cholesterol was found to be at essentially the same level as in normal fetal glands. The fetal adrenal ascorbic acid was depressed by 25%.

Effect of Cortisone (Table 3)

The administration of 30 mg. of cortisone acetate to pregnant rats caused little or no change in the maternal adrenal glands. The response found in the fetal glands was similar to that found in the low dose ACTH and the stress experiments. The fetal cholesterol was increased by 70% while the ascorbic acid level decreased by an amount which was not significant.

In the nonpregnant animal, cortisone caused no change in the cholesterol, but brought about a decrease of the ascorbic acid of 23%.

Pretreatment with cortisone, followed by .16 I.U. of ACTH, did not protect the glands from depletion (not included in Table 3). In fact, the de-

TABLE 2. THE EFFECT OF I.V. ACTH ON MATERNAL AND FETAL ADRENAL CHOLESTEROL AND ASCORBIC ACID

	Adrenal cholesterol					Adrenal ascorbic acid				
	No. of animals	Adrenal cholesterol mg./100 mg.	Per cent change	*T* value	Significance	No. of animals	Adrenal ascorbic acid mg./100 gm.	Per cent change	*T* value	Significance
Non-pregnant—control	20	4.44				12	333			
Non-pregnant—ACTH 100 μg. (0.16 I.U.)	8	3.77	↓ 15	2.36	± P = <.05, >.02	9	215	↓ 36	4.6	+
Non-pregnant—ACTH 1000 μg. (1.6 I.U.)	7	2.86	↓ 35	4.97	+	7	221	↓ 33	4.1	+
Pregnant—maternal control	14	2.57				5	345			
Pregnant—ACTH 100 μg. (0.16 I.U.)	7	1.95	↓ 24	2.15	± P = <.1, >.05	8	185	↓ 46	7.9	+
Pregnant—ACTH 1000 μg. (1.6 I.U.)	10	1.88	↓ 26	2.73	± P = <.02, >.01	12	158	↓ 53	9.4	+
Fetus—control	12*	0.80				4	222			
Fetus—ACTH 100 μg. (0.16 I.U.)	6	1.26	↑ 58	2.99	+	6	182	↓ 18	1.85	− P = >.1
Fetus—ACTH 1000 μg. (1.6 I.U.)	10	0.80	0	0	0	8	134	↓ 40	3.4	+

* Cholesterol determinations only were done on 8 of these letters.

creases in cholesterol (19%) and ascorbic acid (48%) were even greater than when ACTH alone had been given, although the number of experiments of this type was small.

DISCUSSION

Since it is probably justifiable to assume that decreases in adrenal cortical ascorbic acid and cholesterol content indicate adrenal stimulation (review by Sayers, 1950), these data can best be interpreted as showing that stimulation of the pregnant rat's adrenal by cold or by moderate doses of

TABLE 3. THE EFFECT OF CORTISONE ACETATE ON MATERNAL AND FETAL ADRENAL CHOLESTEROL AND ASCORBIC ACID

	No. of animals	Adrenal cholesterol mg./100 mg.	Per cent change	*T* value	Significance	No. of animals	Adrenal ascorbic acid mg./100 gm.	Per cent change	*T* value	Significance
Non-pregnant—control	20	4.44				12	333			
Non-pregnant—cortisone	4	4.34	0	0	0	4	257	↓ 23	6.75	+
Pregnant—control	14	2.57				5	345			
Pregnant—cortisone	8	2.81	0	0	0	8	350	−	−	−
Fetus—control	12	0.80				4	222			
Fetus—cortisone	7	1.37	↑ 70	4.00	+	6	188	↓ 15	1	−

ACTH produces a response comparable to that observed in nonpregnant animals receiving the same degree of stimulation. The response in the fetus not only does not parallel the maternal response, but it is usually in the opposite direction, that is, secretion by the fetal adrenal is inhibited when the mother's adrenal is stimulated. It seems probable that the mater-

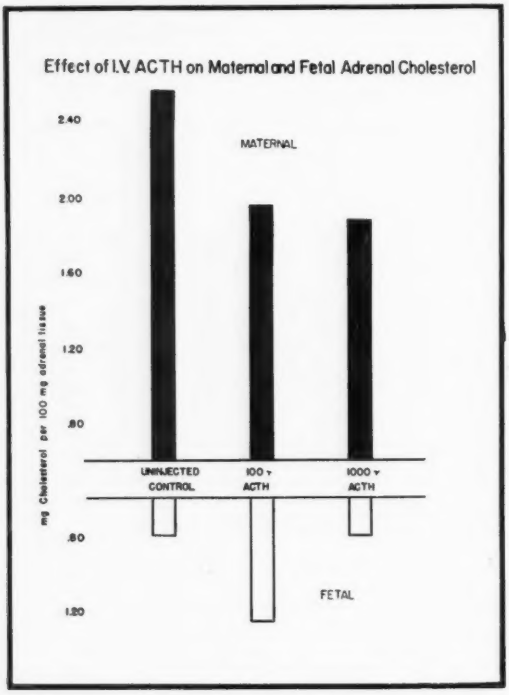

FIG. 3

nal adrenal responded to endogenous ACTH in the stress experiments or exogenous ACTH when this material was administered, but that the ACTH did not cross the placental barrier to stimulate the fetal adrenal. Rather, it appears that adrenal cortical steroids secreted by the mother's cortex crossed the placenta and caused an inhibition of the fetal pituitary-adrenal axis. The administration of a cortical steroid such as cortisone also caused a suppression of fetal adrenal cortical secretory activity, which tends to confirm the view that with moderate degrees of adrenal cortical stimulation, maternal steroids inhibit the fetal adrenal. On the other hand, the administration of large amounts of ACTH produced fetal adrenal stimulation in addition to stimulation of the maternal adrenal. This would indicate that ACTH can traverse the placenta although it appears to do

so only when the level is considerably above those which probably are present under physiological conditions.

Further evidence that ACTH in usual concentrations does not cross the placenta has been presented by Jost (1952). He has shown that when embryos are decapitated *in utero*, the surviving embryo has considerable atrophy of its adrenals. This indicates that the fetal adenohypophysis is

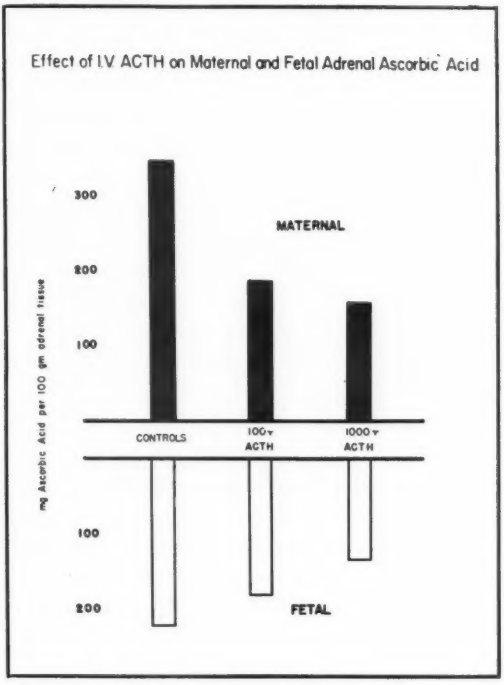

FIG. 4

necessary for maintenance of the normal fetal adrenal and that in the absence of the fetal pituitary, sufficient ACTH does not cross the placenta from the maternal circulation to maintain the fetal adrenal. Jost's data, as well as the results presented in this paper, suggest that in uterine life the pituitary-adrenal axis has functional significance and that the fetal adrenal is responsive to ACTH. Whether or not the pituitary-adrenal axis possesses the same efficiency of response in the fetus as in older animals is not known, but it seems probable that this fetal endocrine function is less effective in light of the work of Jailer, who has found the newborn pituitary-adrenal axis less efficient than that of the adult.

In the few studies of fetal adrenal function following cortisone adminis-

tration it was apparent that cortisone, or some metabolite thereof, entered the fetal circulation quite freely. This is the finding of De Costa and Abelman (1952). That cortisone causes the death of fetuses when it is given to pregnant rabbits might be attributed to the passage of cortisone into the fetuses, although it is not impossible that the cortisone produces changes in the maternal organism which cause fetal death.

The maternal adrenal gland was found to be large and to have a low level of cholesterol. In other words, the maternal adrenal cortex appears to be secreting at a high level. Studies by Anderson and Sperry (1937) led to the same conclusion as have the results of excretion studies in pregnant humans. The pregnant woman's corticosteroid excretion is considerably elevated. The source of these steroids is probably the maternal adrenal and placenta (Johnson and Haines, 1952), but since the fetal adrenal appears to be functional, the possibility exists that some small moiety of the increased corticosteroid might be of fetal origin. Our data also suggest that moderate doses of ACTH given to the pregnant woman would probably not cross the placenta and experience with pregnant patients who have received ACTH has not indicated any harmful effects on the baby, although there is no available evidence as to whether or not the ACTH has passed the placenta.

SUMARY AND CONCLUSIONS

1. Cholesterol and ascorbic acid concentrations of maternal and fetal rat adrenals have been studied.

2. The cholesterol concentration is lower in the maternal adrenal than in the nonpregnant rat's adrenal, average values being 2.4 and 3.5 mg.% respectively. Fetal adrenal cholesterol concentration is .81 mg.%. Ascorbic acid concentration of maternal and fetal adrenals is in the same range as for nonpregnant animals.

3. Exposure to cold or administration intravenously of moderate doses of ACTH caused depletion of maternal adrenal cholesterol and ascorbic acid. Concomitantly, a rise in fetal cholesterol and little or no change in ascorbic acid concentrations occurred.

4. Administration of cortisone to pregnant rats also produced a rise in fetal cholesterol.

5. Administration of large amounts of ACTH caused a depletion of fetal as well as maternal adrenal ascorbic acid and cholesterol.

6. These data suggest that ACTH does not cross the placental barrier of the rat under conditions of moderate stress or moderate dosage of ACTH, but that steroids of maternal adrenal cortical origin do pass the placenta and inhibit the fetal adrenal. It appears that high levels of ACTH in the

maternal rat can enter the fetus and produce a stimulation of the fetal adrenal.

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EFFECT OF PANCREATIC EXTRACTS ON KETONE BODY PRODUCTION OF RAT LIVER

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THE hypothesis that the pancreas affects ketone body production through the elaboration of a hormone other than insulin, is supported chiefly by experiments in which the pancreas is removed from an alloxan-diabetic dog, (Thorogood and Zimmermann, 1945; Rodriguez-Candela, 1945). Removal of the gland usually results in an increased ketosis and decreased insulin requirement in such an animal. Some investigators have sought the explanation of such findings in the metabolic properties of the Pancreatic Hyperglycemic-Glycogenolytic Factor (HGF). This substance, the hormonal nature of which is still uncertain, raises blood sugar by speeding liver glycogenolysis, probably through activation of the enzyme phosphorylase (Sutherland, 1951). Although this property of HGF may account in part for the lowered insulin requirement of the depancreatized alloxan-diabetic dog, compared to the alloxan treated animal retaining its pancreas, few investigations of a possible effect of HGF upon ketone body metabolism have, as yet, been made. Foa and his co-workers have reported (Foa and Weinstein, 1950; Foa, Santamaria, Weinstein, Smith and Berger, 1952) the preparation of an extract, derived from fractions obtained during the manufacture of insulin, which reduces ketosis in the depancreatized dog. These authors are, however, apparently of the opinion that the factors responsible is not identical with HGF. In the present study it was decided to endeavour to prepare "antiketogenic" extracts of the pancreas and then attempt to correlate this property with their content of HGF. Certain extracts of the gland have been shown to depress ketone body production by incubated rat liver slices, provided that extract of the anterior pituitary is also present in the incubation medium. These extracts are also active in raising the blood sugar of the rabbit. In addition, a purified sample of HGF, prepared by the Eli Lilly Company[1] has been shown to depress ketogenesis, in a similar way.

Received for publication March 2, 1953.

This work was assisted by a grant from the National Research Council of Canada. Some of the data reported were presented at a meeting of the Canadian Physiological Society, October 10th, 1952.

[1] A sample of this purified HGF was provided through the courtesy of the Eli Lilly Company.

METHODS

Female rats of the Sprague-Dawley strain, weighing between 70 and 160 gm. were fasted for 12 hours and anesthetized with sodium pentobarbital. The livers were removed, and slices weighing between 60 and 90 mg. (wet) were prepared freehand. These slices were incubated for 2 hours in Krebs-Ringer-phosphate (Umbreit, Burris and Stauffer, 1949) at 37.5° C. and pH 7.4. Each slice was placed in 5.1 ml. of medium and shaken at a rate of 80 oscillations per minute, while a slow stream of moist oxygen was passed through the flask. When appropriate, dried pancreatic or anterior pituitary extracts were dissolved in the medium.

Ketone bodies were determined on the incubation fluid using the method of Rappaport and Baner (1943). Hyperglycemic effects of the extracts were determined by intravenous injection in rabbits, the blood sugar being estimated by the method of Hagedorn and Jensen (1923).

Preparation of the Pancreatic Extracts

Most previous investigators have relied on extraction methods for HGF, closely allied to those used for extracting insulin (e.g. Kazal, Wolfe, Spicer and Barnes, 1950). If the working hypothesis is adopted that the desired material is manufactured by the α cells of the pancreas, then extraction with water rather than acid alcohol should be effective, as the α cell granules are much more water soluble than either the β or zymogen granules (Bensley, 1938). Accordingly, after pilot experiments, the following scheme of extraction was adopted.

Five pounds of fresh ox pancreas were trimmed of fat, minced in a meat grinder and then treated briefly in a "Waring Blendor" with enough water to form a thin suspension (total volume 2500 ml.). This extract was then chilled to 2° C., stirred for 30 minutes and centrifuged. The precipitate so obtained was discarded. The supernatant was filtered with suction through Whatman #4 paper to remove solidified lipid. Using extreme care not to overshoot, the pH was then adjusted to 4.80 by the addition of dilute sulfuric acid solution, and the extract allowed to precipitate overnight at 2° C. It was then centrifuged, yielding precipitate 1P and supernatant 1S. 1P was then suspended in 1200 ml. of sodium hydroxide-glycine buffer at pH 9.5 (2.5 parts 0.1 N sodium hydroxide solution plus 7.5 parts 0.1 N glycine solution), allowed to stand overnight at 2° C. and then centrifuged, yielding precipitate 2P and supernatant 2S. Fraction 2P was then suspended in 750 ml. of water and the pH carefully adjusted to 3.65 by addition of dilute sulfuric acid. Precipitation was allowed to proceed overnight at 2° C. and the extract centrifuged, yielding precipitate 3P and supernatant 3S. Precipitate 3P was then suspended in 250 ml. of 5% sodium chloride solution, stirred for 30 minutes, and centrifuged, yielding precipitate 4P and supernatant 4S. Fraction 4P was discarded and 4S lyophylized without further treatment.

Preparation of Anterior Pituitary Extract.

Anterior pituitary extract was prepared from ox pituitary glands.[2] The anterior lobes were separated by hand, macerated in a "Waring Blendor" with an appropriate amount of 0.9% sodium chloride solution and the coarser particles removed by filtration through cheesecloth. The extract was then lyophilized.

[2] The glands were obtained through the kind cooperation of Canada Packers Limited.

EXPERIMENTS AND RESULTS

In preliminary experiments the rate of ketone body production by untreated liver slices was studied. It was found that under the conditions chosen, production increased in a linear fashion with time, at least up to 2½ hours incubation, with no sign of a plateau in the curve. It was decided that an incubation time of 2 hours would be suitable for the following studies.

In experimental series 1 the various pancreatic extracts, prepared as above, were added to liver slices and the ketone body output compared with untreated controls. Table 1 presents representative results. No significant difference could be detected between the output of the treated and

TABLE 1. EFFECT OF PANCREATIC EXTRACTS ON UNTREATED LIVER SLICES

Treatment of experimental slices		Average production (5 slices each) expressed as μgm. acetone/100 mg. liver	
Extract added	Dosage per slice (mgs.)	Control value[1]	Experimental value[1]
1P	1	86 ± 4	80 ± 3
4S	0.01	86 ± 6	82 ± 6
4S	0.01	124 ± 5	117 ± 8
Lilly HGF	1	133 ± 8	150 ± 5

[1] Mean values are reported together with their standard errors.

untreated slices. It was found convenient to store the extracts in the lyophilized form, weigh out the desired quantity, and dissolve it directly in the medium.

In experimental series 2 (Table 2) anterior pituitary extract was added to both control and experimental slices. The crude extract described above was used in a concentration of 40 mg. of the dried powder per 100 ml. of Krebs-Ringer-phosphate. Under these conditions slices treated with pancreatic extracts 1P and 4S produce an average of 30% less ketone bodies than those treated with anterior pituitary extract alone, with fraction 4S being the most active on a weight basis. Similarly prepared extracts from fresh rabbit liver showed no activity (extracts 1PL and 4SL). Other experiments, which it is unnecessary to report in detail, have shown that fractions 1S, 2S, and 3S are inactive and apparently do not contain appreciable quantities of the "antiketogenic" factor. The purified HGF prepared by the Eli Lilly Company also depresses ketone output, but a larger dose is necessary to demonstrate the effect than when fraction 4S is used. Preliminary experiments have also indicated that while purified growth hormone, or commercial ACTH may take the place of crude anterior pituitary extract, prolactin can not.

TABLE 2. EFFECT OF VARIOUS EXTRACTS ON LIVER SLICES TREATED WITH APE

Treatment of experimental slices		Average production (5 slices each) expressed as μgm. acetone/100 mg. liver		Significance of difference[1]
Extract added	Dose per slice mg.	Controls	Experimentals	
1P	1	115 ± 11	85 ± 5	P 0.05
4S	0.01	117 ± 13	83 ± 5	P 0.05
4S	0.01	136 ± 9	96 ± 7	P 0.01
4S (+GH)[2]	0.01	143 ± 7	120 ± 6	P 0.05
4S (+ACTH)[3]	0.01	151 ± 5	119 ± 6	P 0.01
Lilly HGF	1	164 ± 4	112 ± 6	P 0.01
Lilly HGF	0.01	140 ± 5	156 ± 9	—
1PL (Liver)	1	153 ± 12	161 ± 8	—
4SL (Liver)	1	168 ± 9	171 ± 9	—
Ovalbumin	1	142 ± 6	136 ± 6	—
Ovalbumin	1	106 ± 16	128 ± 8	—
Insulin	1	142 ± 6	136 ± 8	—

Mean values are reported together with their standard errors.
[1] Probability of a chance occurrence of the difference between the means, from Fisher's table of T values.
[2] 0.1 mg. of growth hormone added to each flask instead of anterior pituitary extract.
[3] 0.1 mg. of ACTH added to each flask instead of anterior pituitary extract.

In Table 3 typical blood sugar responses in the rabbit, after injection of fraction 4S, are reported. The desired quantity of dried extract was dissolved in 2 ml. of water and injected in the marginal ear vein. Blood samples were obtained from the other ear. Well-fed animals were used in the tests for hyperglycemic activity, and animals fasted 24 hours in the tests designed to detect possible insulin activity. Well-marked hyperglycemic responses were obtained in the fed animals, and no change observed in the fasted; behaviour typical of HGF. No insulin activity could be detected at the doses employed.

DISCUSSION

The results reported above show that it is possible to prepare insulin-free pancreatic extracts which cause hyperglycemia on intravenous injection and depress the rate of ketone body production by liver. In order to demonstrate the effect on ketone bodies, it is necessary to treat the test

TABLE 3. EFFECT OF FRACTION 4S ON BLOOD SUGAR IN THE RABBIT

Dose injected μgm.	Blood sugar in mgs. % glucose					Nutritional state of rabbit
	Minutes after injection					
	0	10	20	40	60	
500	127	148	156	136	133	Fed
500	138	163	178	168	169	Fed
500	91	91	99	101	109	Previously fasted 24 hrs.
500	118	122	118	117	122	Previously fasted 24 hrs.

system, consisting of rat liver slices incubated in Krebs-Ringer-phosphate, with an extract of the anterior pituitary. The most active fraction prepared (4S) will cause a significant suppression of ketone body production at a dose of 10 micrograms per liver slice. The investigation was undertaken in the hope of demonstrating the existence of a hypothetical ketone body regulating hormone of the pancreas, and as a preliminary assumption, it was supposed that this factor was identical with HGF. However, due to the fact that the problems involved in putting the "antiketogenic assay" on a quantitative basis are as yet unsolved, it is not possible to trace any quantitative parallel between the hyperglycemic and antiketogenic actions of the extracts we have prepared. It can only be stated that purification of the hyperglycemic and antiketogenic factors has followed the same path. Our experiments suggest that fraction 4S is more active in depressing ketone body production than Lilly HGF, but it cannot be claimed that this fact is definitely established. The question of the identity or nonidentity of HGF and the antiketogenic factor must, therefore, remain open. The experiments of Foa *et al.* (1952), on the suppression of ketosis in the depancreatized dog, strongly suggest the occurrence of two separate factors, as these authors report that purified Lilly HGF was ineffective in this respect, whereas other extracts from insulin manufacture were active. It should be noted, however, that their antiketotic extracts provoked a considerable rise in blood sugar.

We have no explanation to offer as to why the addition of anterior pituitary extract to the incubated liver slices is necessary in order to demonstrate the antiketogenic effect of the pancreatic extracts. It might be supposed that the pancreatic extracts are preventing the increased ketone body production that would otherwise be caused by the APE. Doubt is thrown upon this explanation, because it has not been found possible to demonstrate a significant increase in ketone output caused by the addition of pituitary extract, using ovalbumin as a control (see Table 2). The nature of the interaction between the pancreatic and pituitary extracts is, therefore, obscure.

SUMMARY

An investigation has been carried out with the hope of demonstrating a hypothetical ketone body regulating hormone of the pancreas, not identical with insulin. By aqueous extraction of the gland, followed by other purification steps, an extract has been prepared which causes on the average a 30% reduction in the ketone body output of rat liver slices, provided that anterior pituitary extract is also present in the incubation medium. The extract also causes hyperglycemia in the fed, but not in the fasted

rabbit, and exhibits no insulin activity in this species. Methods of preparation and testing of the extract are reported in detail.

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THE EFFECT OF HYPOPHYSECTOMY ON FETAL AND PLACENTAL GROWTH IN THE RAT

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THE original observation by Pencharz and Long (1931) that hypophysectomy after the 11th day of gestation did not interrupt the progress of pregnancy in the rat has been repeatedly confirmed and extended. These investigations, however, did not encompass the effects of pituitary ablation in the pregnant animal on fetal or placental growth. The present study was undertaken to determine whether or not hypophysectomy on the 12th day of pregnancy would, under controlled conditions, influence fetal and placental growth in the rat.

MATERIALS AND METHODS

The animals employed in this study were female rats of the Sprague-Dawley strain, weighing approximately 200 gm. at the time of mating. They were selected for estrus by the vaginal smear technique between 9:00 and 11:00 P.M. and placed with males. The females were segregated the morning of the following day—day 1 of pregnancy. On the 12th day of gestation laparotomies were performed and pregnancy was confirmed in 22 animals. Fourteen of these were hypophysectomized by the parapharyngeal approach immediately following laparotomy and the remaining 8 rats were subjected to sham operations in which the hypophysis was exposed but not removed. 30,000 units of penicillin were administered prophylactically to each animal at the time of operation. Pair feeding[2] (controls against experimentals) was initiated the day following operation. The animals were sacrificed on the 21st day of gestation, the gravid uteri were removed and the fetuses and placentae were trimmed of adhering tissues, blotted on paper toweling and individually weighed on a torsion balance.

Completeness of hypophysectomy was determined by visual inspection of the *sella turcica*.

RESULTS

On the twenty-first day of pregnancy, the hypophysectomized animals could not be distinguished from their sham-operated, pair fed controls by visual inspection. Both groups were active and in apparent good health. It seems clear from the data in Table 1 that the weight gains of the control and hypophysectomized pregnant animals were quite comparable; the

Received for publication March 5, 1953.

[1] Milton Fellow, Harvard University.

[2] Ground Purina Laboratory Chow.

TABLE 1. THE EFFECTS OF HYPOPHYSECTOMY ON MATERNAL WEIGHT AND LITTER SIZE

Experiment	No. animals	Body wt.[1] at operation gm.	Body wt.[2] at autopsy gm.	Wt. change gm.	No. fetuses per litter
Pregnant Control	8	234.5 ± 4.3[3]	264.1 ± 2.9	29.6 ± 5.4	9.4
Pregnant Hypophysectomized	14	241.8 ± 4.8	277.0 ± 7.9	35.2 ± 4.8	9.4

[1] 12th day of gestation.
[2] 21st day of gestation.
[3] Mean ± standard error of the mean.

difference between the recorded values is not significant. Hypophysectomy, likewise, failed to influence litter size when compared with the controls (Table 1).

Hypophysectomy on the 12th day of gestation, however, effected a highly significant decrease of fetal weight as measured on the 21st day of pregnancy (Table 2). Placental weights were also reduced when compared with the controls but the significance of this difference is questionable.

DISCUSSION

The decreased fetal and placental weights observed in females which were hypophysectomized on the 12th day of pregnancy and autopsied on the 21st day cannot be attributed to a reduced maternal food intake since this variable was controlled by limiting the food consumption of the pregnant controls to that of the hypophysectomized animals. In this regard, it should be noted that the weight gain of the two groups was of the same magnitude and that this increase in weight can be accounted for in its entirety by the conceptus. There was, therefore, no net increase in maternal weight in the hypophysectomized group and a small weight loss in the control group. Since litter size and length of gestation in the experimental and control groups, were identical, the influence of these factors on fetal size can also be discounted. The data suggest, therefore, the possibility that the pituitary gland may have a direct influence on fetal and placental

TABLE 2. THE EFFECT OF HYPOPHYSECTOMY ON FETAL AND PLACENTAL WEIGHTS

Experimental groups	Number	Weight gm.	Fisher's t	Fisher's P
Control Fetuses	69	3.76 ± 0.03[1]		
(Hypox.) Fetuses	131	3.26 ± 0.06	7.88	$< < 0.001$
Control Placentae	68	0.498 ± 0.0095		
(Hypox.) Placentae	131	0.467 ± 0.0075	2.58	< 0.02

[1] Mean ± standard error of mean.

size. Positive evidence to this effect has been presented by Teel (1926), Sontag and Munson (1934) and Hain (1933) who obtained heavier young at birth by administering alkaline extracts of anterior pituitary glands to pregnant female rats. These increased birth weights could not be accounted for solely by the increased length of gestation effected by injection of the pituitary extracts.

The remarkable well-being of the hypophysectomized pregnant rat when compared to non-pregnant hypophysectomized animals cannot be completely explained at present. That adrenocorticotropin and thyrotropin production by the placenta is probably not involved has been shown by Greer (1949) and his findings have been confirmed in this laboratory (Knobil, Caton and Shaw, 1952). The possibility that the placenta may produce adrenocortical-like steroids in addition to estrogens, progesterones and the gonadotropins is now under investigation.

SUMMARY

Hypophysectomy of pregnant rats on the 12th day of gestation effected a highly significant decrease in fetal weight and a lesser decrease in placental weight when these were examined on the 21st day of gestation. These changes could not be accounted for by differences in maternal food intake, litter size or length of gestation since these factors were rigidly controlled. The possibility that the pituitary gland may have a direct effect on fetal growth is discussed.

Acknowledgments

The authors wish to thank Mr. Leston B. Nay, Jr. and Mrs. Lorelei W. Parker for invaluable technical assistance.

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ADDENDUM

Since the submission of this manuscript for publication, we have become aware of an article by Rosa M. Campbell, I. R. Innes and H. W. Kosterlitz entitled "Some Dietary and Hormonal Effects on Maternal, Foetal and Placental Weights in the Rat" (*J. Endocrinol.* **9**: 68. 1953). These authors report that hypophysectomy on the 13th or 15th day of pregnancy effected a decrease in fetal weight on the 21st day, but they state that this decrease was not statistically significant. Unfortunately, their data for

fetal and placental weights are not accompanied by statistics of variation although a "considerable scatter of fetal weights" is admitted and this is attributed by the above authors to a possible inaccuracy of sampling.

The discrepancy between our findings and those of Campbell *et al.* is a quantitative one which may indeed be accounted for by a difference in the accuracy of timing with regard to conception and termination of the experiment and the resultant homogeneity of the fetal and placental populations.

CORTICOSTEROID METABOLISM IN LIVER: STUDIES ON PERFUSED RAT LIVERS*

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FOLLOWING the discovery that cortisone possesses beneficial effects in disease processes (Hench *et al.* 1949) studies were undertaken in this laboratory to delineate the transformation products resulting from the perfusion of cortisone and related corticosteroids through organs in an attempt to elucidate the contribution of individual organs to the metabolic pattern of corticosteroid metabolism. In view of its dominant role in a variety of metabolic processes, the liver was selected as the first organ to be studied. Upon perfusion of either cortisone or 11-desoxycorticosterone (DOC) through either rat or rabbit liver, it was found that the added corticosteroid disappeared from the system. While our studies were still in progress, reports from other laboratories appeared which demonstrated that liver slices possess active systems for modifying added cortisone or related corticosteroids. Thus, Paschkis *et al.* (1951) and Louchert and Jailer (1952) found that the glycogenic activity of cortisone markedly declined after incubation with rat liver slices. Schneider and Horstmann (1951, 1952) have reported extensive reduction of Δ^4-3-keto grouping and loss of the ketol side chain of cortisone and a variety of typical corticosteroid hormones, upon incubation with surviving liver slices of the rat, and to a lesser extent with the guinea pig.

Prior to undertaking large scale perfusion studies to obtain sizeable amounts of metabolites sufficient for chemical characterization, studies were undertaken to (a) develop methods for the extraction of corticosteroid metabolites from liver perfusates, (b) study perfusion conditions requisite for the production of cortisone metabolites and (c) to attempt to evaluate the general nature of the metabolites formed at varying intervals of the perfusion period by measuring chemical changes in the functional groupings of the corticosteroid molecule. The results of these studies, wherein cortisone, hydrocortisone, 11-desoxycorticosterone (DOC), 17-hydroxy-11-desoxycorticosterone were employed in liver perfusion are presented in this first paper of a series concerned with corticosteroid metabolism in liver.

Received for publication March 5, 1953.

* Aided in part by U. S. Public Health Service Grant No. RG-2842.

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METHODS

General: In the initial stages of this study, search was made for a convenient *in vitro* system for demonstrating liver enzymatic activity upon corticosteroids. It was observed that rat or rabbit liver perfused with 100 mg. of added cortisone or DOC in 1 liter of various media (homologous citrated blood, heterologous bovine citrated blood or in a Kreb's Ringer phosphate solution containing 6% polyvinyl pyrollidone) recycled some 100 times through the liver completely transformed the added steroid since it disappeared from the perfusate (the charcoal method of Levy *et al.* (1953) was employed) and was not recoverable from the liver. The disappearance of added steroid likewise occurred whether the liver was perfused through either the portal vein or the hepatic vein, so that normal course of hepatic circulation was reversed. The foregoing results indicated that liver tissue perfused under a wide variety of conditions transforms added DOC and cortisone.[1] An *arbitrary* set of perfusion conditions was therefore adopted and the metabolism of corticosteroids was studied in systematic fashion. Our basis for this approach was that once having obtained information for any one set of conditions, it would be possible to use these results as a standard for comparison to evaluate the influence of variables such as species, perfusion media, etc.

Preparation of rat livers for perfusion: Fed male rats weighing 280 to 490 gm. were lightly etherized and exsanguinated by cutting the femoral arteries and veins. A cannula was then inserted into the thoracic vena cava, and the residual blood within the liver was flushed out with physiological saline perfused at a pressure of 40 mm. Hg. The vena cava below the liver was tied off and the liver was then removed. The outflow in this preparation is mainly through the portal vein. All preparations were stored in cold (2–5° C.) physiological saline (generally 5 to 10 minutes) before they were introduced into the perfusion apparatus. The livers weighed 10.8 to 23.2 gm., and were uniformly perfused with non-pulsatile perfusion pressures varying from 40 to 48 mm. Hg., and the hepatic blood flow ranged from 2.4 to 4.5 ml./min./gm. tissue. The degree of edema in these perfused livers (measured by weighing before and at the end of the experiment) is less than 5% of the initial weight. The rate of blood flow in any particular liver was held relatively constant during the entire course of the experiment.

Preparation of bovine blood for perfusion: Citrated bovine blood obtained at a local slaughter house, was collected and stored in the manner previously described (Hechter *et al.*, 1953). On the day of experimentation, the blood was filtered through seven layers of muslin and then introduced into the perfusion apparatus (Hechter, 1948) where further filtration through four layers of china silk was carried out under pressure. During this filtration period, the blood was fully oxygenated with 95% O_2–5% CO_2 and was warmed to 38° by circulation through the apparatus. After this mechanical filtration, corticosteroids were added to the blood in the following manner: An ethanolic solution of corticosteroid (10 mg. per ml.) was added dropwise very slowly to rapidly swirling blood to give a final concentration of 10 mg. steroid per 100 ml. blood containing 1% ethanol. Each steroid employed in these studies had been purified by silica gel chromatography and recrystallization, so that it was homogeneous by paper chromatography.

Recycling of perfusates: In the experiments to be reported, the blood containing corticosteroid was recycled through the liver. Since the volume of perfusate in the apparatus

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[1] This should not be interpreted to indicate that there may not be quantitative or qualitative differences in corticosteroid metabolism between rat and rabbit livers or between different perfusion conditions.

is known, and the blood flow through the liver can be measured, it is possible at any time to calculate the number of times the perfusate has been recycled through the organ. This calculation expresses a "statistical" recycling, since the blood issuing from the organ mixes with the reservoir blood so that some portions of blood are circulated through the organ more frequently than others. The cycle periods referred to in the paper are thus values calculated on this basis, except in the case of the 1 cycle perfusates where the venous effluent was withdrawn directly from the system.

Extraction of corticosteroid metabolites: The nature of corticosteroid metabolites formed in liver were unknown when these studies were initiated. Since the products formed might conceivably be dissimilar to the added steroids, studies were undertaken with radioactive DOC to develop an extraction procedure which would satisfactorily remove the bulk of metabolites from liver perfusates. The DOC samples employed were labeled at either C-3 or C-21.[2]

The first extraction procedure tested was the charcoal extraction developed at the Worcester Foundation (Levy, *et al.* 1953) which involves adsorption of steroids from blood on charcoal and subsequent elution with methylene chloride.

In four consecutive experiments using either C-3 or C-21 labeled DOC, or a mixture of both, essentially similar results were obtained in that while uniformly 90–96% of the radioactivity was recoverable from the control blood sample which was not perfused through liver, only 40–60% of the added radioactivity could be recovered from liver perfusates.

Studies undertaken to search for the missing radioactivity revealed that the perfused liver contained less than 2% of the added counts,[3] and that exhaustive washings of the perfusion apparatus contained neglible radioactivity. Since a second charcoal treatment of the perfusates did not yield significant radioactivity, it appeared that all radioactive compounds capable of being adsorbed by charcoal had been removed by the first charcoal treatment. To test the possibility that radioactive products in liver perfusates were bound by charcoal more firmly than DOC and thus could not be eluted by methylene chloride, charcoal samples from liver perfusates after methylene chloride extraction were systematically eluted with a battery of solvents (ether, pentane, benzene, methanol, and pyridine) but only a small percentage (3–5%) of the missing radioactivity was obtained by these additional extraction procedures.

To obtain further information upon the apparent loss of radioactivity occurring as the result of liver perfusion, we were led to test direct solvent extraction methods. Using first a modification of the ether-chloroform method of Nelson (1952) and later the ethyl-acetate method of A. Meyer (unpublished work) we have been able to recover a good

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[2] All C^{14} DOC samples employed were obtained from Dr. D. Heard, McGill University in the form of the acetate. The material was suitably diluted, saponified, purified by careful silica gel chromatography and recrystallization. On paper chromatography using either propylene glycol-toluene (3 hrs.) or propylene glycol-cyclohexane (24 hrs.) systems, the DOC samples gave only a single spot containing the radioactivity and no other area of the paper possessed significant radioactivity.

[3] It should be mentioned that the values shown for the liver sample are complicated by the fact that the perfusion blood was not washed out of the liver before analysis; nevertheless it is clear that despite inclusion of this complicating factor, the accumulation of radioactivity in the perfused organ was only 2% of the total radioactivity introduced into the system.

portion of the radioactivity, not isolated by the charcoal method. Table 1 presents the results of an experiment where the ether-chloroform method was compared to the charcoal procedure on the same samples of blood. C^{14} labeled DOC, equally labeled at C-3 and C-21 (2,800 cts./min./mg.) was perfused for 1, 5, and 10 cycles, and at each interval

TABLE 1. COMPARATIVE RECOVERY OF RADIOACTIVITY FROM C^{14} DOC* PERFUSATES WITH THE CHARCOAL AND SOLVENT EXTRACTION METHODS

Sample	Charcoal					Ether-chloroform (4:1)				
	Vol. ml.	Counts† input	Residue mg.	Counts† found	% rec.	Vol. ml.	Counts† input	Residue mg.	Counts† found	% rec.
Control	100	28,000	21.7	25,700	92	50	14,000	19.9	13,500	97
1 cycle	100	28,000	16.1	17,211	61	50	14,000	17.4	14,400	100
5 cycle	100	28,000	14.8	16,500	59	50	14,000	16.1	13,800	99
10 cycle	100	28,000	12.0	17,202	61	50	14,000	12.0	11,100	79

* 2,800 cts. per mg. per min.
† Total counts per minute.

a 200 ml. sample was removed for analysis. The perfusates were compared to a control sample containing DOC which had not been perfused through the liver. Half of each sample was extracted with the charcoal procedure in the usual fashion, while 50 ml. of the other portion was extracted directly in the following manner. The blood (without dilution) was extracted 4 times with 2 volumes of ether-chloroform (4:1), and the organic phase was washed with 1/10 volume of water three times, dried with anhydrous sodium sulfate, and brought to dryness *in vacuo*. The residues were weighed and samples counted. It will be seen from Table 1 that the ether-chloroform method quantitatively removes all of the radioactivity from the 1 and 5 cycle liver perfusates and 79% of the activity at 10 cycles, whereas only 60% was recoverable by the charcoal method after 1 to 10 cycles.

To check the possibility that charcoal failed to adsorb radioactive products extractable by ether-chloroform, the charcoal treated perfusate samples of Table 1 were re-extracted with ether-chloroform. The residues thus obtained did not contain significant radioactivity in any case. This finding strongly suggested that the total radioactivity present in perfusates had been completely removed by charcoal but either was (a) only partially eluted by organic solvents, or (b) partially lost as the result of reactions of transformation products upon charcoal surfaces. Using a sensitive low background flow counter (background 6–8 cts./min.) in cooperation with Dr. Manfred L. Karnovsky, at the Biophysical Laboratory, Harvard Medical School, methylene chloride extracted charcoal samples from perfusates (10–15 mg. per planchette) were directly counted to determine whether the charcoal samples contained the missing radioactivity. It was found that the control sample (wherein 92% of the radioactivity had been recovered by methylene chloride elution) had approximately the same number of total counts (1800 cts. per min.) as the 5 and 10 cycle perfusate samples (containing 1700 and 2800 cts. per min. respectively). By calculation, 8000 and 10,000 cts. per min. from an added 28,000 cts. per min. are not accounted for in the charcoal samples from 5 and 10 cycle perfusates respectively. These observations therefore suggest that certain corticosteroid transformation products lose radioactivity on charcoal, perhaps by loss of the radioactive carbon atoms at C-21 and/or C-3. It should be emphasized that the results are complicated by

the fact that the expected radioactivity, if present, is so diluted by the charcoal that accurate counts are not possible in this range, due to factors such as possible quenching effects of the charcoal.

Effect of liver perfusion upon corticosteroids: Having developed suitable solvent extraction methods for the extraction of DOC transformation products, and evidence that DOC does not accumulate to any significant degree in the liver during perfusion, studies were undertaken to determine the rate of disappearance of the added corticosteroid, and the nature of the transformation products formed. For this purpose, DOC, cortisone, 17-hydroxycorticosterone, and 17-hydroxy-11-desoxycorticosterone (Reichstein's S) were perfused through rat livers and samples removed after 1, 5, and 10 cycles. In some experiments with cortisone, values were obtained at 3 cycles as well. In all experiments, the ether-chloroform extraction method was employed. Three experiments with each corticosteroid were carried out with the following experimental design: Blood (containing 10 mg. steroid per 100 ml.) was prepared and mixed for 30 minutes in the perfusion apparatus. A 50–100 ml. sample was then removed and extracted as control No. 1. A 150 ml. sample was removed, transferred to another pump and circulated in the absence of a liver for the duration of the liver perfusion experiment (40–150 min.) to determine any possible influence of blood incubation alone upon the added steroid (Control, No 2). Usually, 50 ml. samples of the perfusates and Control 1 and 2 (equivalent to 5 mg. of the added steroid) were extracted, and a variety of tests carried out upon aliquots of the residues to evaluate changes in various functional groups of the corticosteroid molecule. For the ketol side chain, the blue tetrazolium (B.T.) method of Chen and Tewell (1951) and the formaldehydogenic steroid (F.S.) periodate oxidation method of Daughaday *et al.* (1948) (slightly modified in both cases) was employed. The latter method measured glycols as well as ketols, thus large differences in the titres between B.T. and F.S. might reflect possible formation of glycols. Changes in the Δ^4-3-ketone grouping was measured by determining the U.V. absorption at 240 mμ. using a Beckman model D.U. spectrophotometer (purified methanol as solvent). Measurements were made in the range 220 to 280 mμ. at intervals of 5 or 10 mμ. To check the possible production of 17-ketosteroids arising from degradation of the corticosteroid side chain, the Zimmermann chromogen (Z) at 515 mμ was measured using the modifications of Holtorff and Koch (1940). The ketonic groupings of typical corticosteroids give yellow Z chromogens with maxima in the range 420–480 mμ which are about $\frac{1}{4}$–$\frac{1}{5}$ that produced by dehydroepiandrosterone (pink Z chromogens) when read at 515 mμ. Thus any significant production of true 17-ketosteroids should be accompanied by a rise in the Z titre. In all of the above determinations, the values obtained were corrected from a blank equivalent to same volume of blood extract under test (obtained by extracting a sample of blood to which no corticosteroid had been added). The standards used in the above tests were the specific steroids used in the perfusions. This is particularly important in the case of the B.T. determination because it has been found that depending upon the batch of B.T. employed and slight temperature differences, the optical density per μM steroid, with the 4 corticosteroids employed may vary as much as two-fold.

To determine the absolute disappearance of the corticosteroid added and the appearance of new compounds as evidenced by spot tests, the paper chromatography technic of Zaffaroni *et al.* (1951) was employed. Extract equivalent to 2.5 mg. of the added corticosteroid was applied to 5.0 cm. paper strips and developed in varying solvent systems for various periods of time depending upon the steroid added.

The following spot tests were employed to determine the presence of new compounds:

a) Blue tetrazolium (3-3′-dianisole-bis-4, 4′-(3,5-diphenyl) tetrazolium chloride) for

determination of ketols in the manner described by Zaffaroni *et al.* (1951), for triphenyl tetrazolium (red tetrazolium).

b) Dinitrophenylhydrazine, which reacts with Δ^4-3-ketosteroids and saturated 3-ketones (Zaffaroni *et al.* 1951).

c) Zimmermann reaction for detection of 17-ketosteroids on paper strips (H. Rosenkrantz, 1952).

d) Mineralite U.V. lamp which detects Δ^4-3-ketosteroids as darkened areas was also utilized (R. Haynes, unpublished work). It has not been established that the darkening observed is necessarily specific for this grouping.

The systems employed for the various steroids were as follows:

17-hydroxycorticosterone: chloroform-formamide for 8 hours.

cortisone: chloroform-formamide for 4 hours.

17-hydroxy DOC: toluene-propylene glycol for 8 hours.

DOC: toluene-propylene glycol for 3 hours.

Each of these systems moves the steroid involved 15 to 25 cm. from the starting line. The zone containing the added steroid was determined by appropriate spot tests, position on the chromatogram, and the U.V. lamp. The steroid was then eluted with methanol-chloroform (1:1) and estimated by the Chen and Tewell B.T. method. The possible presence of less polar steroids with each type of perfusion was tested by suitable chromatography of the "overflows" obtained from the original chromatogram. Thus in the case of cortisone and hydrocortisone, the chloroform overflow was dried, and partitioned by toluene-propylene glycol for 8 hours. The toluene overflow was then fractionated on cyclohexane-propylene glycol for 4 hours. In the case of DOC and 17-hydroxy DOC, the toluene overflows were fractionated by cyclohexane-propylene glycol for 24 hours, and then the cyclohexane overflow was fractionated by cyclohexane-propylene glycol for 4 hours.

Rate of disappearance of added corticosteroids: Table 2 presents the complete details of three experiments with cortisone. It will be seen that the rate of corticosteroid disappearance (in terms of μg. steroid disappearance per gm. liver per minute of perfusion) during the *first* circulation (1 cycle) averages 97 μg./min./gm., and then declines to about $\frac{1}{5}$ of this value as the blood is recirculated for 3, 5, or 10 cycles. The lowering of rate observed after the first cycle, when the perfusate contains *products of reaction* as well

TABLE 2. THE TRANSFORMATION OF CORTISONE BY PERFUSED RAT LIVER*

Expt.	Liver wt.	Sample	Cortisone content	Perfusion time	Blood flow	Vol. of perfusate at each period	Cortisone utilized	
							Total	Rate
	g.		mg./100 ml.	min.	ml./min.	ml.	mg./period	μg./min./g.
1	17.2	Control #1	9.6	—				
		Control #2	9.3	(Incubated 82′)				
		1 Cycle	5.7	10 (0–10)	55	550	22	123
		5 Cycle	3.1	36 (10–46)	45	450	12	20
		10 Cycle	0	36 (46–82)	55	400	12	20
2	23.0	Control #1	9.2	—				
		Control #2	8.5	(Incubated 70′)				
		1 Cycle	4.4	9 (0–9)	45	400	19	92
		5 Cycle	2.1	25 (9–34)	48	300	7	12
		10 Cycle	0	25 (34–69)	50	250	2	4
3	20	Control #1	9.5	—				
		Control #2	9.3	(Incubated 196′)				
		1 Cycle	6.6	32 (0–32)	52	1650	48	78
		3 Cycle	5.4	43 (32–75)	72	1550	19	22
		5 Cycle	4.4	53 (75–128)	53	1400	22	14
		10 Cycle	2.0	44 (128–196)	51	450	11	12

* Perfused at an initial concentration of 10 mg. per 100 ml.

as unreacted cortisone, may result from (a) the lowering of substrate concentration as cortisone is utilized, (b) depletion of some component of the hepatic enzymatic apparatus involved, or (c) the accumulation of certain primary or secondary reaction products in the system which act to inhibit the rate. The fact that the initial rate is essentially unchanged despite variation in the period of first cycle perfusion varying from 9 to 32 minutes (compare Expt. 1 to 3, Table 2) suggests that factor (b) is not of major importance in this regard. Although further experiments are clearly needed to establish the influence of initial cortisone concentration upon the initial rate to check factor (a), it appears that *after* the first cycle the utilization of cortisone remaining in the system is not markedly dependent upon the cortisone concentration in the perfusion medium.

Similar results were obtained with the other corticosteroids tested, in that there was a high initial rate of utilization during the first cycle, and a lower rate upon recycling 5 and 10 times. These results are shown in Table 3 where the average results with hydrocortisone, DOC, and 17-hydroxy DOC are compared to those obtained with cortisone. In all cases, the rates of utilization are of the same order of magnitude, and further experimentation would be necessary to determine whether the apparent differences noted between individual corticosteroids in this extremely small number of experiments are really significant.

TABLE 3. THE TRANSFORMATION OF VARIOUS CORTICOIDS BY PERFUSED RAT LIVERS*

Corticoid	No. of experiments	Av. initial rate	Av. secondary rate
		μg./min./g.	μg./min./g.
Cortisone	3	97	15 (22–4)†
17 hydroxycorticosterone	3	123	16 (25–8)
DOC	3	120	12 (20–10)
17 hydroxy DOC	3	130	14 (21–12)

* Perfused at an initial concentration of 10 mg. per 100 ml.
† Range.

These hepatic utilization rates for corticosteroids cannot be explained in terms of hepatic storage since it will be recalled from our studies with C^{14} labeled DOC (cf. Table 1) that 90–96% of the added radioactivity is extractable from perfusates. This means that certainly in the case of DOC, and presumably with the other corticosteroids, the bulk of the corticosteroid disappearing is converted to metabolic products circulating in the perfusate.

Disappearance of functional groups of corticosteroids relative to the corticosteroid substrate: The basic pattern of changes observed with cortisone, hydrocortisone, DOC and 17-hydroxy DOC were essentially similar. Table 4 shows the results obtained in the cortisone experiments, where the disappearance of functional groupings is measured relative to the disappearance of the added cortisone. All results were measured in terms of cortisone equivalents and are expressed as percentage recovery.

It will be seen from Table 4 that:

(a) Incubation of cortisone with blood alone does not lead to significant loss of added corticosteroid, measured as cortisone by paper chromatography, or by the various tests for functional groups.

(b) The loss of the Δ^4-3-ketone grouping (measured by U.V. absorption at 240 mμ) closely follows the curve of cortisone disappearance. This suggests that the bulk of transformation products formed from cortisone no longer possess the conju-

TABLE 4. THE DISAPPEARANCE OF VARIOUS FUNCTIONAL GROUPINGS RELATIVE TO CORTISONE, AT VARYING PERIODS OF PERFUSION

Expt.	Sample	Per cent recovery				
		Cortisone	B.T.	F.S.	U.V.	Z
1	Control #1	96	91	93	85	88
	Control #2	93	90	91	88	85
	1 Cycle	57	62	65	52	48
	5 Cycle	31	57	60	27	35
	10 Cycle	0	40	55	13	23
2	Control #1	92	90	92	94	91
	Control #2	85	100	88	98	88
	1 Cycle	44	63	67	48	55
	5 Cycle	21	60	57	40	50
	10 Cycle	0	51	43	20	40
3	Control #1	95	96	92	88	88
	Control #2	93	97	88	86	88
	1 Cycle	66	87	89	75	75
	3 Cycle	54	68	72	57	52
	5 Cycle	44	66	70	48	36
	10 Cycle	20	64	66	31	25

B.T. = blue tetrazolium.
F.S. = formaldehydogenic steroid.
U.V. = absorption at 240 mu.
Z = Zimmermann.

gated unsaturated system in ring A. The presence of minor amounts of products retaining this grouping is also indicated.

(c) The disappearance of the ketol side chain as measured by B.T. is wholly dissimilar from that of cortisone. At 1 cycle, the B.T. recovered is in large part accounted for by cortisone. After 5 and 10 cycles, when most of the added cortisone has disappeared from the medium, the perfusate still contains B.T. material, and to the extent of 40–50% of the original value. This suggests that the products formed during recycling in the main retain the ketol side chain. The loss of the ketol side chain in the remaining products, by a possible reduction of ketol to glycol, is excluded as a major factor since the F.S. values (which measures both ketols and glycols) are essentially similar to the B.T. values (which measures ketols but not glycols).

(d) The Zimmermann chromogen measured at 515 mμ declined steadily during the perfusion following U.V. indicating the net loss of ketone groups. This would appear to exclude the possible production of typical 17-ketosteroids from cortisone as *major* end products.

These features of cortisone perfusion, hold for the other corticosteroids, and are in full conformity with the results of paper chromatography. The findings observed will be discussed under the following headings:

(a) *Influence of incubation of steroid in blood, in the absence of liver:*

Incubation in blood at 38° for 40 to 200 minutes, does not markedly transform cortisone or the other steroids tested, the bulk of the corticosteroid added being recovered unchanged. With each steroid, however, there is evidence by paper chromatography, for the development of trace amounts of new ketols, more polar than the corticosteroid substrate. This is evident in the Control No. 1 sample which represents a 30 minute incuba-

tion period, and is more pronounced in the Control No. 2 samples. The nature of these more polar ketols formed in blood is unknown, and definitive chemical characterization will be difficult since they are present in only trace amounts. However, they do not appear to react with DNPH, or give a U.V. reading on paper. It thus seems probable that they no longer possess the Δ^4-3-ketone grouping in the added corticosteroid. As an incidental finding, it may be mentioned that the incubation of DOC in beef blood gives rise to spots, more polar than DOC, which give cortisone-like blue coloration with iodine, in contrast to the yellow-brown spots usually produced by Δ^4-3-ketosteroids (Zaffaroni *et al.*, 1951).

(b) *Disappearance of the conjugated unsaturated system:*

The conclusion from the U.V. data that the major products formed from corticosteroid during perfusion no longer possess the Δ^4-3-ketone grouping is fully confirmed by analysis of papergrams. None of the papergrams formed reveal compounds during liver perfusion which react with DNPH to give orange or yellow spots. However, when larger samples of liver perfusates are worked up, the presence of trace amounts of new Δ^4-3-ketosteroids and 3-ketones is indicated by spot tests with DNPH.

(c) *Appearance of new steroid ketols:*

With all of the corticosteroids perfused there is evidence, particularly at 5 and 10 cycles, and to lesser extent at 1 cycle, of new steroid ketols, which react with B.T. but which show no reaction with DNPH. These spots are observed with all of the corticosteroids studied and are generally more polar than the starting material. However, in all cases, chromatography reveals trace amounts of ketol less polar than the added corticosteroid substrate. The number of new ketol spots arising from liver perfusion varies with each compound, and with the conditions of chromatography. If large amounts of perfusates are worked up, evidence from paper chromatographic analysis would suggest that a veritable galaxy of compounds is formed with all of the steroids studied. With cortisone and DOC, the steroids most extensively studied with large amounts of perfusate (i.e. 100 mg.), a conservative estimate of the number of new compounds formed would be 20, as evidenced by spot tests.

DISCUSSION

It is important at the outset to emphasize that liver perfusion, as carried out under our conditions, has been employed as a convenient biochemical tool for evaluating certain aspects of corticosteroid metabolism in hegatic tissue. It is *not* necessarily a system for studying liver action upon corticosteroids under physiological conditions. With this limitation in mind it is apparent that the physiological significance of our results cannot be assessed in either a quantitative or qualitative sense, until appropriate *in situ* experiments are carried out on living animals.

Rate of transformation of corticosteroids under in vitro conditions.: With all of the corticosteroids studied, the initial transformation rate is about 100 μg. per minute of perfusion per gm. (wet weight) of liver, during the first cycle, and this decreases during recycling to about 15–25 μg./min./gm. The magnitude of this degree of corticosteroid transformation may be appreciated by extrapolating these values to human liver, not as an example of what occurs *in situ*, but merely for illustrative purposes. Assuming

a human liver weight of 1500 gms., this would correspond to the transformation of 150 mg. of cortisone or 17-hydrocorticosterone per minute or 9.0 grams per hour, on the basis of our initial rates of corticosteroid transformation. If the secondary lower transformation value is assumed, (15 μg./min./gm.) this would correspond to 22.5 mg./minute or 1.35 gm./hour. These values are so extraordinarily high that an independent check would be highly desirable. Fortunately *in vitro* data on cortisone metabolism in rat liver slices is available from the work of Schneider and Horstmann (1952). Using cortisone concentrations of 250 μg./ml. (or concentrations 2.5 times greater than ours) they report that the disappearance of cortisone (evaluated by loss of U.V. at 240 μm. which we have shown mirrors cortisone disappearance closely), averages 3.0 μg./mg. tissue (wet weight) per 3 hours of incubation at 38° C.

According to these authors the curve of cortisone disappearance in time is essentially similar to the results found with DOC in an earlier paper (Schneider and Horstmann, 1951). Examination of the DOC time curve of Schneider and Horstmann (1951) reveals that 50% of the total reaction which occurs at 3 hours is achieved in 15 minutes, and that at 1 hour the reaction is about 73% of the value at 3 hours. From their combined data it becomes possible to calculate the rates of reaction at 15, 60 and 180 minutes, and the rate of cortisone disappearance between these various periods. Table 5 shows these calculated rates of cortisone disappearance in rat liver slices, relative to rat liver perfusion experiments. It will be seen that the data on rat liver slices give essentially similar values for cortisone disappearance as that obtained with liver perfusion, both for the initial and secondary phases of the reaction. The combined findings of slice work and perfusion afforded a striking demonstration, using completely independent techniques, that the extraordinary capacity of rat liver to transform added cortisone is a real phenomenon.

TABLE 5. COMPARISON OF *in vitro* UTILIZATION OF CORTISONE BY RAT LIVER (SLICES VS. PERFUSION)

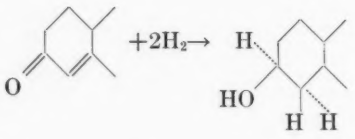
	Period of liver incubation	Rate of utilization
	min.	μg./gm./min.
Slices*	0–15	100
	0–60	36
	0–180	17
	15–60	14
	60–180	7
Perfusion	1 Cycle	97
	5–10 Cycle	15

* Recalculated from data of Schneider and Horstmann (1951, 1952).

Schneider and Horstmann (1952) have likewise studied the comparative rates of metabolism of the various adrenocortical hormones studied here in rat liver slices. They find that both hydrocortisone and cortisone are metabolized to approximately the same degree, while DOC and 17-hydroxy DOC are significantly more resistant (ca 30%) to reduction of the Δ^4-3-ketone grouping. While our perfusion results (cf. Table 3) did not reveal such differences between 11-oxygenated and 11-desoxycorticosteroids, the number of experiments in our series was not sufficient to establish any small but significant differences with the precision of accuracy possible with slices.

It should be emphasized that the high rates of corticosteroid disappearance observed with perfused rat liver, illustrate only hepatic capacity for corticosteroid transformation under unphysiological conditions using 10 mg. steroid per 100 ml. blood. If as seems likely, the rate of hepatic transformation of corticosteroid is dependent upon the concentration of corticosteroid in the blood, it is apparent that under physiological circumstances, the rate of corticosteroid transformation would be markedly lowered. Studies are necessary to quantitatively evaluate the hepatic transformation rates *in vivo*. Although different techniques and analytical procedures were involved, the data of Nelson (1952), using dogs, not rats, indicates that cortisone, administered intravenously, disappears rapidly from the blood stream under *in vivo* conditions, and that the liver was the sole organ of those studied, wherein there was a significant A.-V. difference.

Corticosteroid hormones as hydrogen acceptors: Under our conditions of liver perfusion, it has been calculated that the initial rate of corticosteroid utilization is about 100 μg./gm./minute, and the secondary rate of utilization is about $\frac{1}{5}$ of this value. If one considers this to occur exclusively as the result of reduction of the Δ^4-3-ketone grouping, for every mole of corticosteroid disappearing, 2 moles of H_2 are taken up according to the following reaction:



On this assumption, one may calculate that 6000 ug. or 16.6 uM of corticosteroids require 33.2 μM of H_2 per gm. liver per hour of incubation to effect the reduction of Δ^4-3-ketone grouping. The magnitude of this hydrogen uptake may be appreciated by considering the hydrogen transport involved in glucose oxidation in liver slices. Chernick and Chaikoff (1951) have measured the rate of CO_2 production from C^{14}-labeled glucose in rat

liver slices and find glucose utilization values of 267 μg. or 1.4 μM./hour/gm. liver. From the equation, $C_6H_{12}O_6+6O_2 \rightarrow 6\ CO_2+6\ H_2O$ it is possible to calculate the hydrogen transferred from glucose and eventually accepted by O_2 to form water, and this corresponds to 8.4 μM./hour/gm. In other words, corticosteroids at a concentration of 100 μg. per ml. (or .027 μM./ml.) in the presence of the appropriate enzyme system could theoretically accept almost 4 times as much hydrogen, as would be available from the complete oxidation of glucose in hepatic tissues. The physiological significance of this finding remains to de demonstrated. Nevertheless it is clear that the Δ^4-3-ketone grouping present in steroid hormones may be regarded as a powerful naturally occurring hydrogen acceptor, which could thereby reduce the oxygen consumption of tissues. The findings that a Δ^4-3-keto steroid like testosterone, at relatively high concentration, reduces the oxygen consumption of tissue slices (including liver) (Kochakian, 1951) (Gordan *et al.*, 1951) may be an expression of this activity.

Biological activity of corticosteroid transformation products: The finding that rat hepatic tissue transforms cortisone and 17-hydroxycorticosterone, rapidly and at significant rates under *in vitro* conditions raises an interesting question with respect to the biological activity of these corticosteroids. Both cortisone and 17-hydroxycorticosterone are only slightly less effective *per os* than by parenteral injection (Freyburg *et al.* 1950) (Boland and Headley, 1951). It has been generally assumed that steroid administered by mouth following intestinal absorption are shunted directly into the liver via the portal vein, and that the lowered effectiveness of orally administered hormones such as estradiol, estrone, testosterone, progesterone and DOC is a reflection of the greater degree of hepatic inactivation occurring as the result of oral administration. If cortisone is adsorbed into the circulation from the gut in a manner similar to other steroid hormones, the results in man would indicate that the *anti arthritic activity* of both cortisone and 17-hydroxycorticosterone is not subject to marked hepatic inactivation. Nor is this apparent resistance of a particular cortisone activity to liver inactivation confined solely to man. The thymolytic activity of cortisone in mice is approximately the same when the hormone is administered into the spleen (drained mainly by the portal vein) or injected systemically (deAndino and Rivero-Fontan, 1951). It would therefore appear from the *in vivo* studies that certain cortisone activities are apparently not inactivated markedly by the liver. If the *in vitro* data cited here is qualitatively valid for other species, *e.g.* men and mice, it would appear that there is a contradiction between the *in vivo* and *in vitro* findings. One explanation to resolve this apparent discrepancy is to *assume* that *some of the products formed in liver metabolism possess anti-arthritic, thymolytic and possibly*

certain of the other biological activities associated with cortisone administration. The foregoing tentative conclusion does not assume that *all* of the biological activities associated with cortisone, are necessarily resistant to hepatic inactivation. The work of Paschkis *et al.* (1951) and of Louchart and Jailer (1952) clearly demonstrates that the *glycogenic* activity associated with cortisone is markedly lowered by incubation with rat liver slices.

It has been considered by many that the activity of oral cortisone may be due to its transformation in the body to 17-hydroxycorticosterone. It should be emphasized, however, that our studies, as well as those of Schneider and Horstmann (1952), demonstrate that 17-hydroxycorticosterone is metabolized by liver tissue as rapidly as is cortisone. If the considerations which led us to regard cortisone action in arthritis as possibly due to hepatic transformation products are applied to 17-hydroxycorticosterone, the activity of this steroid would likewise be due to transformation products, and a simple conversion of cortisone to 17-hydroxycorticosterone would not suffice to explain the oral activity of cortisone.

SUMMARY

1. Methods for extracting transformation products resulting from corticosteroid perfusion through isolated perfused rat livers are described.

2. The rate of transformation of cortisone, 17-hydroxycorticosterone, desoxycorticosterone, and 17-hydroxydesoxycorticosterone by perfused rat liver has been determined. At corticosteroid levels of 10 mg. per 100 ml. of blood, liver transforms about 100 μg. per minute per gm. of liver tissue. Upon recycling, when the initial substrate concentration has decreased and products of the initial reaction accumulate in the system, the initial rate of transformation declines to about 15–20 μg. per minute per gm. liver.

3. Corticosteroid metabolism in isolated perfused rat livers appears to follow a basically similar pattern, with all of the corticoids studied. In all cases, the transformation products are, in the main, reduced compounds which no longer retain the Δ^4-3-ketone grouping. Of these, some retain the ketol side chain while others lose the characteristic side chain of corticoids.

4. Some of the physiological implications of these findings have been discussed.

Acknowledgements

We wish to thank E. Gobiel, M. Feinstein and E. Scully for their valuable technical assistance.

We also wish to thank Dr. Max Tishler, Merck & Company, Rahway, New Jersey, for the cortisone and hydrocortisone used in these studies. The desoxycorticosterone and 17-hydroxy-11-desoxycorticosterone was generously supplied by Dr. E.Oppenheimer, Ciba Company, Summit, New Jersey.

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THE INFLUENCE OF DECIDUAL TISSUE UPON PSEUDOPREGNANCY[1]

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THERE seems to be a difference of opinion regarding the influence of decidual tissue upon the duration of pseudopregnancy in rats and mice. Ershoff and Deuel (1943) and Peckham and Greene (1948) found that deciduomata extended the period of pseudopregnancy in the rat which is at variance with the results obtained earlier by Astwood and Greep (1938) and also with the observations by Kamell and Atkinson (1948) on mice. It seems now that this lack of agreement probably was due to differences in size of deciduomata as Olsen *et al.* (1951) have found that pseudopregnancy in rats is prolonged beyond its normal duration in proportion to the amount of decidual tissue present. The present paper includes an extension of these observations on the quantitative relationship between the amount of decidual tissue and the length of pseudopregnancy as well well as information contributing to an explanation of the reaction.

PROCEDURES

Pseudopregnancy was induced by electrical stimulation of the uterine cervices of 100-day-old rats during estrus (Greep and Hisaw, 1938). Four days after cervical stimulation, the endometrium of one or both uterine cornua of each animal was traumatized by inserting a burred needle through the tubal sphincter and passing it down the uterine lumen to the cervix. By withdrawing the needle slantwise, the antimesometrial surface was scratched throughout its entire length. As a result, the decidual response involved the entire endometrium from cervix to tubal junction. In other animals one to ten threads were placed transversely through one or both uterine horns. Seventy-two hours after uterine traumatization an exploratory laparotomy was performed to verify the presence of decidual tissue. Vaginal smears were made daily until the animals came into estrus,

Received for publication March 17, 1953.

[1] This investigation was aided in part by a grant from the National Institutes of Health, United States Public Health Service, administered by Professor Frederick L. Hisaw.

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TABLE 1. PROLONGATION OF PSEUDOPREGNANCY ASSOCIATED WITH THE PRESENCE OF MASSIVE DECIDUOMATA

Treatment	Number of rats	Pseudopregnancy in days
Normal Pseudopregnancy	31	13.0 ±0.3[2]
Normal Pseudopregnancy[1]	12	12.8 ±0.2
1 cornu[1] traumatized[3]	20	18.5 ±1.4
1 cornu traumatized[3]	9	18.9 ±1.4
2 cornua[1] traumatized[3]	18	19.9 ±2.9

[1] Laparotomized on day eight.
[2] Standard deviation.
[3] Antimesometrial surface of uterine lumen was scratched from tubal sphincter to cervix.

marking the termination of pseudopregnancy, and then were continued to determine the regularity of ensuing estrous cycles.

RESULTS AND DISCUSSION

The mean length of pseudopregnancy following cervical stimulation in our colony of closely inbred rats is 13.0 days. However, when the uteri of these animals are traumatized either unilaterally or bilaterally throughout their entire length five days after cervical stimulation, the mean length of pseudopregnancy is prolonged to 18.5 days and 19.9 days respectively (Table 1). Animals subjected to laparotomy, and in which the uteri were not traumatized, showed no extension of the length of pseudopregnancy.

Since it was possible to obtain a prolongation of pseudopregnancy with massive deciduomata, we proceeded to a quantitative study of the effects of varying amounts of decidual tissue upon the length of pseudopregnancy. When a single decidual reaction was induced by transfixing one cornu with a loosely-tied thread of 3-0 surgical silk, the animals remained pseudopregnant for 15 days. The length of pseudopregnancy for animals having two, three, and four threads in one uterine horn was 16.3, 17.1 and 17.7

TABLE 2. INFLUENCE OF THE NUMBER OF DECIDUOMATA ON THE LENGTH OF PSEUDOPREGNANCY

Treatment	Number of rats	Pseudopregnancy in days
1 thread in 1 cornu	11	15.2 ±2.1[2]
1 thread in 1 cornu[1]	13	15.0 ±2.3
2 threads in 1 cornu[1]	14	16.3 ±2.2
3 threads in 1 cornu[1]	15	17.1 ±2.3
4 threads in 1 cornu	10	17.5 ±2.2
4 threads in 1 cornu[1]	24	17.7 ±2.4
6 threads; 3 in each cornu[1]	11	19.8 ±1.9
8 threads; 4 in each cornu[1]	15	20.4 ±2.1
10 threads; 5 in each cornu[1]	12	21.4 ±2.4

[1] Laparotomized on day eight.
[2] Standard deviation.

days respectively. Animals having 6, 8 and 10 deciduomata evenly divided between the horns remained pseudopregnant for 19.8, 20.4, and 21.4 days respectively. (Table 2 and Fig. 1.) Again it was found that exploratory laparotomies three days after uterine traumatization were without effect on the length of pseudopregnancy.

A series of experiments in which laparotomies and hysterectomies were performed show quite definitely that such procedures are without effect on the length of pseudopregnancy. The removal of non-traumatized uteri from pseudopregnant rats or complete hysterectomy on the 9th day of

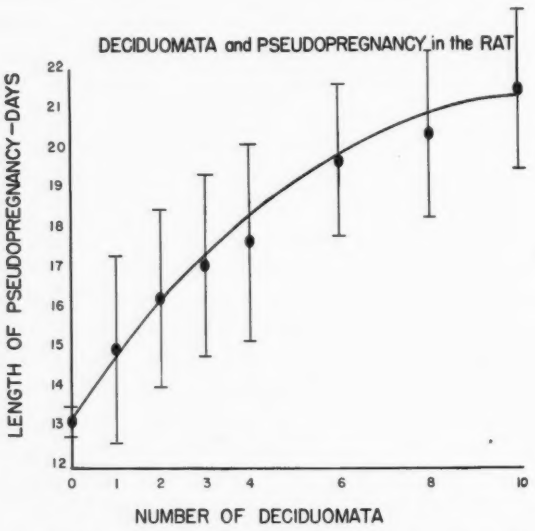

FIG. 1. The quantitative relationship between the number of deciduomata induced by transfixing the uterine cornua with loosely-tied loops of 3-0 surgical silk and the length of pseudopregnancy in days. The brackets above and below each point on the curve represent the limits of the standard deviations.

pseudopregnancy in rats with unilaterally or bilaterally traumatized uteri resulted in pseudopregnancies the average length of which was statistically indistinguishable from the normal. On the other hand, when hemi-hysterectomy was performed on rats whose uteri were bilaterally traumatized, pseudopregnancy was approximately of the same length as that in non-hysterectomized rats having a single traumatized cornu, the arithmetical means being 19.0 and 18.5 days respectively (Table 3).

The rat seems to differ from certain other laboratory mammals with respect to the effect of hysterectomy. Loeb (1923) reported "hysterectomy pseudopregnancy" in the guinea pig and a similar condition has been de-

scribed for the rabbit (Asdell and Hammond, 1933; Sessums and Murphy, 1933; Chu, Lee and You, 1945). It appears that in these two species hysterectomy induces a condition of pseudopregnancy by prolonging the functional life of the corpora lutea. In contrast with this, Long and Evans (1922) and Murphy (1933) did not observe any effect of hysterectomy on the estrous cycle of rats. Other workers, however, have reported that in rats hysterectomized between the fourth and the seventh day of pregnancy the corpora lutea tend to persist and vaginal estrus does not appear until the usual time of parturition (Bradbury, 1937; Hechter, Fraenkel, Lev and

TABLE 3. HYSTERECTOMY AND PSEUDOPREGNANCY

Treatment	Number of rats	Pseudopregnancy in days
Hysterectomized controls	15	12.0 ±1.5[2]
2 cornua[1] traumatized; bilaterally hysterectomized on day 9	10	13.0 ±1.1
1 cornu[1] traumatized; bilaterally hysterectomized on day 9	14	12.7 ±1.8
2 cornua[1] traumatized; unilaterally hysterectomized on day 9	15	19.0 ±2.4
1 cornu[1] traumatized[3]; not hysterectomized	20	18.5 ±1.4

[1] Laparotomized on day 8 of pseudopregnancy.
[2] Standard deviation.
[3] Antimesometrial surface of uterine lumen was scratched from tubal sphincter to cervix.

Soskin, 1940). Whatever the underlying causes may be for these species differences and the seemingly inconsistent reports, our experiments definitely indicate that for the rat hysterectomy has no effect on the length of pseudopregnancy.

The prolongation of pseudopregnancy under the conditions of our experiments seems to be correlated with the presence of decidual tissue. An examination of decidual tissue on successive days of a prolonged pseudopregnancy shows that an orderly sequence of events takes place in decidual development and involution. The reaction occurs first in the antimesometrial region, then in the mesometrial area, and lastly in the mesometrium. Involution occurs in this same order so that at the termination of prolonged pseudopregnancy the only apparently functional decidual tissue is the metrial gland (Velardo *et al.*, 1952).

The ovaries during prolonged pseudopregnancy have corpora lutea of similar appearance to those observed during normal pseudopregnancy, and their histochemistry indicates the continuation of luteal function. It was also found that ovulation is suppressed in the presence of massive decidual reactions (Dawson and Velardo, 1952). It may be significant that the two functional structures that persist under these conditions are the metrial gland and the corpora lutea.

The vaginal smears indicate a condition comparable with diestrus

throughout prolonged pseudopregnancy. Histological sections show that a well mucified vaginal mucosa is maintained until immediately before the estrus marking the termination of pseudopregnancy. At this time a zone of cornification appears below the layers of mucified cells lining the vaginal lumen (Velardo *et al.*, 1952).

SUMMARY

The presence of massive deciduomata prolongs pseudopregnancy in the rat until about the normal time of parturition. This prolongation is related quantitatively to the amount of decidual tissue present. Ovulation and vaginal cornification are suppressed while it is apparent that the functional life of the corpora lutea is extended. Hysterectomy or simple laparotomy is without effect upon the normal duration of psuedopregnancy.

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COMPARISON OF THE EFFECT OF 11-KETOPROGESTERONE, 11α-HYDROXYPROGESTERONE AND 11β-HYDROXYPROGESTERONE UPON THE GLYCOSURIA OF THE PARTIALLY DEPANCREATIZED RAT

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VERY large doses of progesterone (50 to 100 mg. per rat per day) cause exacerbation of diabetes in the partially depancreatized, force-fed rat. The diabetogenic potency of 11-ketoprogesterone is greater than that of progesterone (Ingle, Beary and Purmalis, 1953). The data of the present study show that the diabetogenicity of 11β-hydroxyprogesterone is as great or greater than that of 11-ketoprogesterone and each of these compounds is much more potent than 11α-hydroxyprogesterone. The first demonstration that 11-ketoprogesterone is diabetogenic and that 11α-hydroxyprogesterone is relatively inactive was in unpublished experiments by Dr. R. O. Stafford and Mr. W. P. Baker, Department of Endocrinology, The Upjohn Research Division.

METHODS

Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until the metabolic studies were begun. The animals were partially depancreatized at a weight of 275 to 280 gms. After diabetes was established all of the rats were placed in metabolism cages and force-fed a medium carbohydrate diet (Ingle, 1952) by stomach tube each morning (8:30 to 9:30 A.M.) and afternoon (4:15 to 5:00 P.M.). The techniques and diet were modifications of those described by Reinecke, Ball and Samuels (1939). The test substances were ground into a fine suspension in peanut oil and were administered by subcutaneous injection twice daily. The animals were kept at a temperature of 74 to 78° F. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and preserved with toluene. Urine glucose was determined by the method of Shaffer and Williams (1935).

The steroid compounds were obtained from the Department of Chemistry of the Upjohn Company. The compounds 11-ketoprogesterone and 11β-hydroxyprogesterone were derived from 11α-hydroxyprogesterone which was obtained by the microbiological oxidation procedure recently developed in these laboratories (Peterson, *et al.*, 1952).

Received for publication March 19, 1953.

EXPERIMENTS AND RESULTS

Experiment 1 (Fig. 1) involved 5 moderately diabetic rats which were observed during a control period of 14 days. Each rat was treated with 11α-hydroxyprogesterone in doses of 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mg. daily for 5 days per dose. Experiment 2 (Fig. 1) involved 6 moderately diabetic rats given 11α-hydroxyprogesterone in doses of 5, 10, 20, and 40 mg. daily for 7 days per dose. At dosages up to 15 mg. per rat per day there was a small, not clearly significant, suppression in glycosuria and

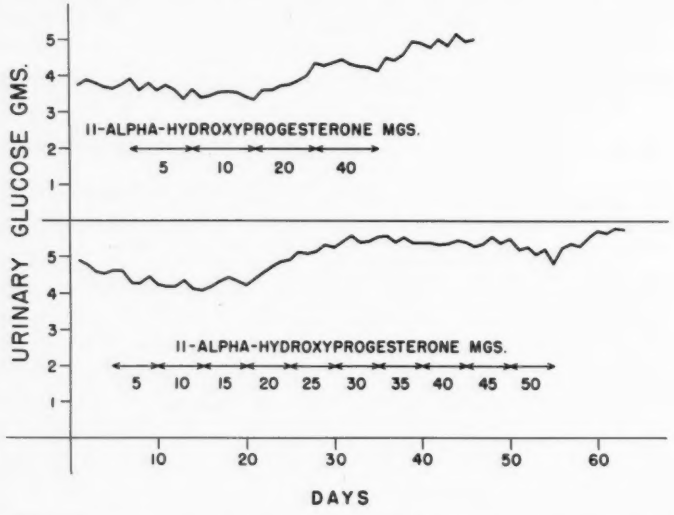
URINARY GLUCOSE GMS.
11-ALPHA-HYDROXYPROGESTERONE MGS.
5 10 20 40
11-ALPHA-HYDROXYPROGESTERONE MGS.
5 10 15 20 25 30 35 40 45 50
10 20 30 40 50 60
DAYS

FIG. 1. Glycosuria of partially depancreatized rats given 11α-progesterone. Averages based upon 5 and 6 rats per group.

thereafter a gradual but small rise. When the injection of steroid was stopped there was no decrease in glycosuria within the 7 to 10 days that the animals were observed.

Experiment 3 (Fig. 2) involved 16 moderately diabetic rats. Following a control period of 14 days, 7 rats were treated with 11-ketoprogesterone and 9 rats were treated with 11β-hydroxyprogesterone. The dosages were 1, 2, 4, 8, and 16 mg. per rat per day for 7 days. The animals were observed for 14 days after the injections were stopped. Doses of 1 and 2 mg. daily were ineffective but doses of 4 mg. per rat per day and greater caused exacerbation of the glycosuria to an extent which was related to the size of the dose. The average increase in urinary glucose reached a higher peak in the rats treated with 11β-hydroxyprogesterone than with the corresponding ketone but the significance of the difference was not established.

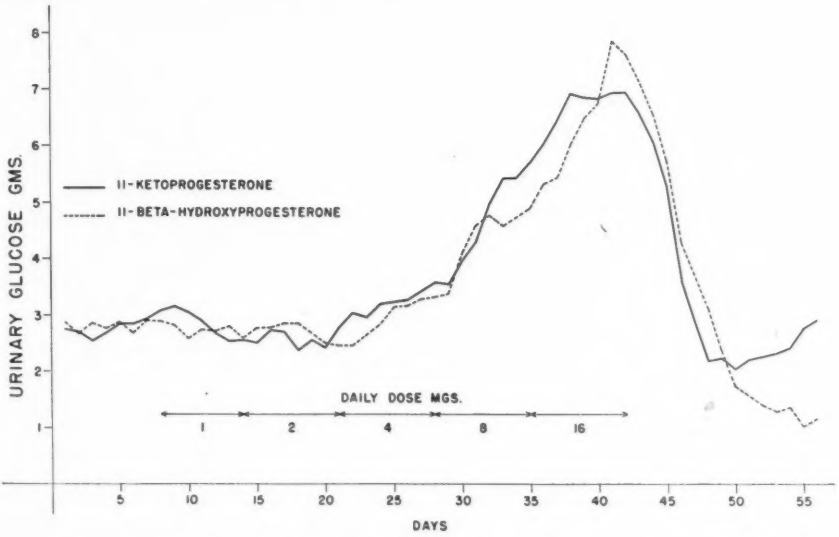

FIG. 2. A comparison of the effects of 11-ketoprogesterone (7 rats) and 11β-hydroxyprogesterone (9 rats) upon glycosuria. Averages.

After stopping the injections the depression of glycosuria below the initial control values was greater with the 11β-hydroxy- than with the 11-keto-compound. It is possible that the degree of suppression of glycosuria following the stopping of injections represents the extent of adrenal cortical atrophy and decreased secretion of cortical hormones but this was not studied.

Experiment 4 (Fig. 3) involved one adrenalectomized, partially depancreatized rat which was maintained on 1 ml. (activity equivalent of 0.1 mg. of hydrocortisone) of adrenal cortical extract per day. Following a control period of 14 days the rat was given 11β-hydroxyprogesterone in doses of 1, 2, 4, 8, and 16 mg. per day for 3 days per dose and was observed for an additional 16 days after the injections were stopped. During the injections there was a striking exacerbation of the diabetes which gradually subsided to preinjection levels when the injections were stopped.

DISCUSSION

The diabetogenicity of the adrenal cortical steroids is enhanced by the presence of an oxygen function at position 11 of the steroid nucleus (Ingle, 1942). This is also true of 11-keto- and 11β-hydroxyprogesterone. The adrenal cortical steroids having an 11β-hydroxy group are more potent than their 11-keto analogs. Our data suggest but do not prove that 11β-

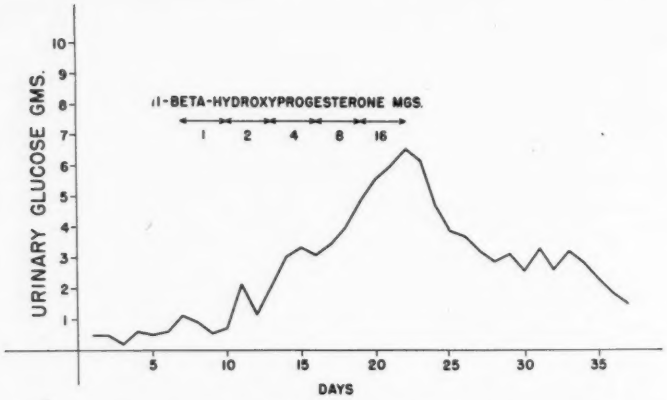

FIG. 3. Exacerbation of diabetes in an adrenalectomized-partially depancreatized rat treated with 1 mg. daily of adrenal cortical extract during the experiment.

hydroxyprogesterone is more potent than 11-ketoprogesterone. Analogs of the natural hormones which bear an 11-hydroxy group in the unnatural (alpha) configuration are relatively inactive. It is not certain that 11α-hydroxyprogesterone caused the small increase in the level of glycosuria in experiments 1 and 2 since there was no decrease when the injections were stopped. The second control period should have been continued for a longer time since delayed absorption from oil depots of crystals may have persisted after the injections were stopped.

The compounds 11-ketoprogesterone and 11β-hydroxyprogesterone are capable of causing some atrophy of the adrenal cortices of the rat. If adrenal cortical insufficiency ensued for this reason the diabetes of the rat should have been ameliorated but this did not occur to a significant extent at any dose. The diabetogenic effect of these compounds is not mediated by the adrenal cortices since it is manifest in their absence (Fig. 3, this paper; Ingle, Beary, and Purmalis, 1953).

SUMMARY

Partially depancreatized force-fed rats were injected with doses up to 50 mg. per rat per day of 11α-hydroxyprogesterone with only a small increase in the level of glycosuria. The compounds 11-ketoprogesterone and 11β-hydroxyprogesterone are significantly more potent in respect to diabetogenicity and this effect is manifest in either the presence or absence of the adrenal glands.

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GLYCOGEN DEPOSITION IN DIFFERENT SKELETAL MUSCLES INDUCED BY CORTISONE AND ESTRADIOL[1]

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TESTERONE, growth hormone, and certain adrenal cortical hormones are known to increase the deposition of glycogen in the skeletal muscles of the rat. Testosterone propionate (1) prevents the loss of muscle glycogen which occurs ordinarily in the fasting hypophysectomized rat (glycostatic effect), (2) increases muscle glycogen in gonadectomized rats (Leonard, 1952 a, b). Growth hormone, besides exhibiting glycostatic effects, increases muscle glycogen in normal rats (Russell and Wilhelmi, 1950; Illingworth and Russell, 1951). Adrenal cortical hormones or extracts of the type which influence carbohydrate metabolism will increase the level of glycogen in the muscles of adrenalectomized and hypophysectomized rats (Long and Katzin, 1938; Russell and Craig, 1939; Long, Katzin and Fry, 1940). In normal fasting rats and mice, these cortical hormones do not increase the deposition of muscle glycogen unless the animals are fed glucose during the period when the hormone is acting (Long, Katzin and Fry, 1940). In all the experiments noted above, with the exception of those involving testosterone, the gastrocnemius and the diaphragm have been used almost exclusively as representative skeletal muscles.

In the experiments employing testosterone, it was observed that the glycogen levels of different skeletal muscles studied did not increase to the same degree (Leonard, 1952 a, b). In order to determine whether a differential response of muscles is the rule for other hormones as well, studies were made on the effect of cortisone acetate on glycogen deposition in several muscles of the rat. In addition, experiments were made using estradiol benzoate in order to re-evaluate previous results of studies of the influence of estrogens on the deposition of muscle glycogen, which were reported negative (Griffith, Marks and Young, 1941; Janes and Nelson, 1942 a; Long, 1942; Walaas, 1952).

METHODS

Adult male and female rats of the Long-Evans strain were selected for uniformity of age and body weight within each experiment. Normal, gonadectomized and hypophy-

Received for publication March 28, 1953.

[1] This work was supported by a grant from the National Research Council, Committee on Problems of Sex.

sectomized animals were employed. Estradiol benzoate,[2] dissolved in peanut oil, was injected subcutaneously in doses of 250 μg. per day in the female rats and 375 μg. per day in the heavier male rats. Cortisone acetate[2] suspended in water was administered subcutaneously in doses of 2 mg. per day and, in one experiment, desoxycorticosterone acetate[2] was given at the same dose level. Control animals received either oil or water. All injections were given once daily for 3 days previous to autopsy.

The animals were starved for 24 hours preceding autopsy. The skeletal muscles selected for glycogen determinations were the *rectus femoris* and a portion of the abdominal muscles taken from rats of both sexes; the cremaster of the males; and the diaphragm of the females. The methods for anesthetizing the rats, removing the muscles, and determining glycogen are described in detail in a previous paper (Leonard, 1952b). The glycogen is reported here as mg. of glucose per 100 gm. muscle, wet weight.

RESULTS

Effect of cortical hormones on hypophysectomized rats. In experiments employing hypophysectomized rats, a period of one week elapsed before injections of the hormones were begun.[3] When cortisone acetate was administered to hypophysectomized rats, an increase in the concentration

TABLE 1. THE EFFECT OF CORTICAL HORMONES AND ESTRADIOL BENZOATE ON THE GLYCOGEN LEVELS IN SKELETAL MUSCLES OF MALE RATS

Experiment	Glycogen (mg. per cent)*					
	Rectus femoris		Abdominals		Cremaster	
	Exper.	Control	Exper.	Control	Exper.	Control
Cortisone in hyp'sect. male	*393 ± 17*	229 ± 9	*424* ± 21	368 ± 10	424 ± 17	454 ± 15
Cortisone in castrated male	*428 ± 22*	334 ± 14	†527 ± 24	463 ± 16	509 ± 18	471 ± 15
Cortisone in normal male	*472 ± 10*	378 ± 16	481 ± 17	465 ± 13	521 ± 25	528 ± 16
Desoxycorticosterone in hyp'sect. male	285 ± 18	250 ± 15	372 ± 13	348 ± 10	357 ± 16	354 ± 28
Estradiol in hyp'sect. male	287 ± 9	256 ± 15	339 ± 7	322 ± 10	417 ± 18	408 ± 16
Estradiol in castrated male	*425 ± 14*	372 ± 15	506 ± 17	463 ± 23	†513 ± 12	468 ± 15

* The averages are based on the results from 10–12 rats in each instance and are presented with the standard error of the mean.

Results in italics indicate the difference of this value from the control is statistically significant (P = .02 or less).

† P = .05; this value was considered of doubtful significance in these experiments.

of glycogen was observed in the *rectus femoris* and abdominal muscles in both sexes and in the diaphragm muscles of the females (see Tables 1 and 2 for the data of these and subsequent experiments). The cremaster muscle, however, failed to respond to the hormone.

[2] Appreciation is herewith acknowledged to the Schering Corporation, Bloomfield, New Jersey for the donation of estradiol benzoate and desoxycorticosterone acetate, and to Merck & Co., Rahway, New Jersey for the donation of cortisone acetate.

[3] It was established that the glycogen levels of these muscles from hypophysectomized rats were not maintained at a normal level after a 24-hour period of fasting. If the hypophysectomized rats were fed up to the time of autopsy, the glycogen levels in these muscles were similar to those of intact fasting rats.

It was also noted that more glycogen was deposited in the *rectus femoris* than in the abdominal muscles in both the male and female rats treated with the hormone. However, if the basic levels of glycogen in the leg and abdominal muscles of the control animals were compared, it was observed that the abdominal muscles contained more glycogen. In the females, the net increase in glycogen in the leg and abdominal muscles, induced by the hormone, was greater than in the males.

Winter and Thomson (1944) reported the failure of desoxycorticosterone acetate to raise the level of glycogen in the skeletal muscles of normal and castrated rats. In contrast to this, Schuman (1940) was able to increase the glycogen content of the leg muscles of castrated rabbits to the level found

TABLE 2. THE EFFECT OF CORTISONE AND ESTRADIOL BENZOATE ON THE GLYCOGEN LEVELS IN SKELETAL MUSCLES OF FEMALE RATS

Experiment	Glycogen (mg. per cent)*					
	Rectus femoris		Abdominals		Diaphragm	
	Exper.	Controls	Exper.	Controls	Exper.	Controls
Cortisone in hyp'sect. female	*485 ± 26*	263 ± 14	*464 ± 17*	365 ± 18	*324 ± 18*	219 ± 16
Cortisone in normal female	*519 ± 23*	417 ± 12	*613 ± 22*	524 ± 14	358 ± 11	337 ± 14
Estradiol in hyp'sect. female	*267* ± 10	207 ± 9	319 ± 12	289 ± 13	269 ± 17	237 ± 10
Estradiol in spayed female	*474 ± 17*	370 ± 15	*465 ± 16*	406 ± 18	*359 ± 14*	273 ± 11

* The averages are based on the results from 10–17 rats in each instance, and are presented with the standard error of the mean.

Results in italics indicate the difference of this value from the control is statistically significant (P = .02 or less).

in normal controls. The glycogen in the muscles of the castrated rabbits had already been depressed as a result of the loss of the male hormone. Since the fasting hypophysectomized rat has a very low level of muscle glycogen, theoretically, it should be sensitive to hormones capable of increasing muscle glycogen. In experiments in which desoxycorticosterone acetate was given to the hypophysectomized males, the results showed that this hormone did not cause a significant increase in glycogen in these muscles (Table 1).

Effect of cortisone on normal and castrated rats. When normal male rats were injected with cortisone, it was observed that only the *rectus femoris* muscle showed an increase in glycogen; the abdominal and cremaster muscles failed to respond. However, when normal female rats were treated similarly, both the *rectus femoris* and the abdominal muscles showed an increase in glycogen but the diaphragm, in this instance, failed to respond.

Previously, it was demonstrated that castration lowered the glycogen content of these muscles in the male rat, although not as severely as that which follows hypophysectomy (Leonard, 1952 b). On the assumption that

this condition would increase the sensitivity of the muscles to cortisone, rats which had been castrated two weeks previously, were injected with the hormone. The results showed that although the glycogen in the *rectus emoris* was increased above that of the controls, the net amount of glycogen deposited was no greater than that which occurred in the same muscle in normal male rats. The abdominal muscles of the treated castrated rats showed a slight increase in glycogen which, statistically, was questionably significant and the cremaster again failed to respond.

Effect of estradiol benzoate on gonadectomized rats. Considering that the mechanism of action of estrogens in raising blood sugar and liver glycogen is believed to be mediated by adrenal cortical stimulation and the fact that cortical hormones increase muscle glycogen, it is difficult to understand why previous attempts to increase muscle glycogen with estrogens failed. The following experiments were made to determine if estradiol benzoate would raise muscle glycogen. Ten male rats were castrated and injected with 20 μg. of the hormone daily for 2 weeks, beginning on the day of operation. Ten castrated males served as controls. A small net increase in glycogen occurred in all three muscles (actually the net amounts were between 46 and 49 mg. %) but this increase was not statistically significant for any muscle. The results are not included in the table.

The experiment was repeated on male rats which had been castrated for two weeks and were then injected with 375 μg. of estradiol benzoate daily for three days (Table 1). The net increase in the amount of glycogen deposited in all three muscles as a result of hormone treatment was small and no greater than that observed in the previous experiment. In the *rectus femoris*, however, the degree of variability of the results was so small, that statistically this increase became significant. The increase in the cremaster muscle was of doubtful significance and in the abdominal muscles, it was definitely negative.

The response to estradiol benzoate in the female differed from that in the male. When female rats were injected with this estrogen (250 μg./day for 3 days) beginning three weeks after spaying, a substantial increase in muscle glycogen occurred (Table 2). The response obtained in the abdominal muscles was not as great as in the leg or diaphragm, however. The female appeared to be more sensitive than the male to this effect of estradiol.

Effect of estradiol on hypophysectomized rats. Although previous investigations revealed that estrogens failed to influence carbohydrate metabolism in the absence of the adrenals or pituitary gland, it was decided to repeat these experiments and examine the same muscles which were employed in these experiments. Estradiol benzoate, injected into hypophysectomized male rats one week after the operation, failed to increase

the glycogen in any of the muscles. When the hormone was given to hypophysectomized female rats, a small but statistically significant increase in glycogen occurred in the *rectus femoris* muscle only. This amounted to a 29% increase.

Body weights of the rats were observed during the course of the experiments but particular attention was given to the changes which occurred from the day hormone injections were begun to the day of autopsy. In all experiments except two, the average loss in body weight of the treated animals was greater than that which occurred in the controls. The two exceptions were in hypophysectomized male rats injected with estradiol and desoxycorticosterone. It was noted in these two instances that none of the muscles of the treated animals showed an increase in glycogen.

DISCUSSION

The results reported above show that cortisone was able to increase the glycogen level in some skeletal muscles but not in others. The *rectus femoris* muscle always responded but the cremaster never did. Previously, it was shown that testosterone increased the glycogen content of both these muscles although, quantitatively, not to the same extent (Leonard, 1952b).

Quantitative differences in the ability of these several muscles to store glycogen under the influence of cortisone was evident throughout these experiments. More glycogen was deposited in the *rectus femoris* than in the abdominal muscles in every case. For example, cortisone administered to hypophysectomized female rats increased the glycogen content of the leg muscle by 84% and of the abdominal muscles by 27%. When normal male rats were used, glycogen was increased by 24% in the leg muscle while no increase occurred in the abdominal muscles.

Cortisone appeared to be more effective in increasing the muscle glycogen levels in hypophysectomized rats than in rats with their pituitary glands intact. The low level of muscle glycogen of the fasting hypophysectomized rats permits those hormones, which by direct action increase muscle glycogen, to exhibit their effects maximally. In most instances, cortisone was able to restore the level of muscle glycogen of fasting hypophysectomized rats to that observed in fasting normal animals. The exceptions were the abdominal muscles of the female and the cremaster. It has been stated that adrenal cortical hormones are not as effective as some anterior pituitary extracts in maintaining muscle glycogen at a high level in hypophysectomized rats (Long, Katzin and Fry, 1940). The present experiments show, however, that if adequate dosages and a sufficient length of time are permitted for cortisone to act, some muscles can maintain a normal level of glycogen. This observation concurs with that of Herring and Evans (1943).

It was demonstrated that estradiol benzoate, administered to spayed female rats, increased the glycogen content of the skeletal muscles. The hormone was markedly less effective in the castrated males and in fact only the leg muscles gave a positive response. Several investigators have reported the failure of estrogens to increase muscle glycogen in either normal or gonadectomized rats although increases in blood sugar and liver glycogen occurred (Griffiths, Marks and Young, 1941; Janes and Nelson, 1942 a; Long, 1942; Walaas, 1952). A possible explanation for the conflicting results may reside in differences, in the period of injection, in the dosages used, as well as in selection of the proper muscle and sex of the animal. Throughout these experiments, the muscles of the female rat appeared more sensitive than those of the male to estradiol as well as to cortisone.

Estradiol was observed to increase glycogen in the diaphragm of the spayed females whereas cortisone failed to do so in this muscle in the normal female. At first sight, these results seem inconsistent. However, there was some indication in the data that spaying possibly lowered the basic level of glycogen in the diaphragm. Thus, when estradiol was injected, the endogenously produced cortical hormone was able to raise the glycogen level to normal. The failure of the diaphragm of the normal female to respond to cortisone could be explained by assuming that the muscle was already responding maximally to the endogenously produced cortical hormone and that the addition of cortisone, under these conditions, was unable to induce a greater-than-normal deposition of glycogen.

It is difficult to explain the small but statistically significant increase in glycogen which occurred in the *rectus femoris* muscle of the hypophysectomized females given estradiol. Possibly the small increase is of no particular consequence or it may reflect a very weak glycostatic effect of estradiol for this particularly sensitive muscle. The fact that estrogens may influence carbohydrate metabolism without the necessary intervention of the adrenal cortex has received some support from Ingle (1943, 1944). These observations in no way negate the concept that estrogens influence carbohydrate metabolism primarily by way of the adrenal cortex (Long, 1942; Janes and Neslon, 1942 b).

The precise method by which the cortical hormones increase muscle glycogen is not clearly understood. The mobilization of blood sugar thus permitting a redistribution of glucose to the muscles may not be the only factor involved. There is good reason to assume that some direct action of cortisone upon the muscle cells takes place and that there exists a certain degree of specificity between the hormone and a particular skeletal muscle. The recent discovery of Kerppola (1952) that cortisone inhibits the enzyme phosphorylase which is one of the essential enzymes in the formation and breakdown of glycogen, offers an opportunity to investigate the mechanism

of action of cortisone and other hormones effecting glycogen storage in different skeletal muscles.

SUMMARY

Estradiol benzoate and cortisone acetate were injected into normal, gonadectomized and hypophysectomized rats of both sexes to determine the effect on the glycogen levels in the following skeletal muscles: the *rectus femoris*, the abdominal muscles, the cremaster of the male, and the diaphragm of the female.

In all experiments, cortisone increased the glycogen content of the *rectus femoris* but failed to do so in the cremaster. The increase in muscle glycogen, induced by cortisone, varied quantitatively in the different muscles. Under similar conditions, more glycogen was deposited in the muscles of the female than in those of the male.

Estradiol increased the storage of glycogen in all three muscles of the spayed female rats and only to a slight extent in the leg muscle of the castrated males. No increase in glycogen was observed in the muscles of the hypophysectomized rats treated with estradiol except for a small but statistically significant increase in the sensitive *rectus femoris* muscle of the female.

The results emphasize that not all skeletal muscles respond alike in depositing glycogen under the influence of hormones.

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NOTES AND COMMENTS

THE EFFECTS OF ADRENOCORTICOTROPIN ON THE WHITE LEGHORN COCKEREL AND CAPON[1,2]

INTRODUCTION

The effects of adrenocorticotropin (ACTH) on the rat and other mammals has received considerable attention in recent years. It is well known that this hormone produces an increase in size and secretion of the adrenals which usually results in a depression of body growth (Evans, Simpson, and Li, 1943; and Asling *et al.*, 1951). It has also been demonstrated that ACTH treatment results in regressive changes in the testes and secondary sex organs of the rat (Li and Evans, 1947 and Baker *et al.*, 1950). The results of studies on the effects of this hormone on the secondary sex organs of castrates have been somewhat conflicting. Davidson (1937) and Nelson (1941) reported that the weights of the seminal vesicles and prostate were larger in castrates treated with ACTH. Baker *et al.* (1950) reported that this treatment produced no change in the seminal vesicles of the castrate rat. Since all of the previous work has been done on the mammal, it was of interest to study the effects of ACTH on the chick.

MATERIALS AND METHODS

Single comb white leghorn cockerels and capons were used throughout these experiments. All birds were wing banded and animals which were caponized were operated on the 19th day of age. The body weights and comb factors (height in mm. times length in mm. divided by 2) were determined prior to the first injection and the animals were assigned to series so that the average comb factors and body weights were as uniform as possible.

The comb factors and body weights were determined at the time of autopsy (40 days) so the gains could be calculated. The testis, comb, adrenal, and pituitary weights were also determined at the time the animals were sacrificed.

The data in Table 1 were obtained from intact animals and capons injected subcutaneously from 20 to 39 days with Armour's ACTH[3] preparation 146 RS3[4] in 0.2 ml. of normal saline. The results in Table 2 were obtained from chicks injected from 1 to 3 days of age. These animals were kept from food and water for the duration of the experiment. A total of five subcutaneous injections were given, the first of which was approximately 18 hours post-hatching. The remaining injections were given at 12 hour intervals and the animals were sacrificed about 12 hours following the last injection.

RESULTS AND DISCUSSION

It is apparent from the data in Table 1 that 0.25 mg. of ACTH per day caused a significant depression of body growth. This effect is probably a result of increased adrenal secretion although there was only a slight increase in adrenal weight. This effect on body growth is comparable to that reported for the rat.

Received for publication March 14, 1953.

[1] Contribution No. 504 from the Department of Zoology, Indiana University, Bloomington, Indiana.

[2] From part of a thesis presented to the faculty of the graduate school of Indiana University in partial fulfillment of the requirements for the Doctor of Philosophy degree in Zoology.

[3] The ACTH used in these experiments was kindly supplied by Dr. Irby Bunding of Armour and Co., Chicago, Illinois.

[4] This preparation has approximately 3.65 I.U. per mg.

TABLE 1. THE EFFECTS OF 0.25 MG. OF ACTH ON THE COCKEREL AND CAPON

No.	Dose mg.	Body wgt. gain	Testis wgt. mg.%	Comb wgt. mg.%	C.F. gain*	Adrenal wgt. mg.%	Pituitary wgt. mg.%
			Cockerels				
14	0.00	301 gm.	29.31	403	131	14.21	0.94
14	0.25	261 gm.†	35.47	623†	187†	16.39	1.15†
			Capons				
12	0.00	228 gm.	—	112	53.42	16.78	1.84
10	0.25	181 gm.†	—	140	63.65	17.88	1.99

* Comb factor gain per 100 gms. body weight gain.
† Significant at the 1% level.

It is of interest to note that the comb growth in the cockerels treated with ACTH was significantly greater than that of the controls. This is in striking contrast to the atrophy of the secondary sex characters reported to occur in the rat treated with this hormone (Baker, Schairer, Ingle, and Li, 1950; and Li and Evans, 1947). This effect is, however, similar to the masculinization often reported to occur in humans under ACTH therapy. It should be pointed out that there was no testis atrophy in the treated birds. This is also in contrast to the results of ACTH treatment in the rat in which there occurs an atrophy of the testes.

There are several possible explanations to account for the increase in comb growth of the cockerels. One is that the adrenals of the chick are capable of secreting androgens. Another is the possibility that the ACTH used contained some gonadotropins. A third possible explanation is that the treatment caused an increased secretion of endogenous gonadotropins.

The first possibility is ruled out by the fact that the ACTH produced no significant increase in comb growth of the capons. The second alternative was tested by assaying the ACTH for gonadotropins using the day old chick method as described by Byerly and Burrows (1938) and Breneman (1945). It is obvious from the data in Table 2 that the testis weights are not increased by the ACTH. These results therefore indicate

TABLE 2. THE EFFECTS OF ACTH ON THE TESTIS WEIGHTS OF BABY CHICKS

Number animals	Dosage in mg.	Testis weights in mg.
15	0.00	7.08
12	0.50	6.98
12	1.00	7.10

that there were no gonadotropins present unless the contaminant was able to produce an increased secretion of androgen without a corresponding increase in testes weight. This seems unlikely since Breneman (1938) showed that the gonadotropic factor (F.S.H.) which caused an increase in androgen secretion also produced the greatest increase in testes weight. This would also tend to eliminate the possibility that the increased comb growth was due to an increase of endogenous gonadotropins.However, the fact that the pituitaries of the treated animals were larger would indicate that there was an increase in gonadotropin secretion.

SUMMARY AND CONCLUSIONS

The administration of 0.25 mg. of ACTH per day for 20 days to White Leghorn cockerels and capons caused a significant reduction in body weight gain. There also occurred an increase in comb growth in the intact animals but not in the capons. This treatment failed to produce atrophy of the testes which has been reported to occur in the rat. The pituitaries of the intact animals injected with ACTH were significantly larger than those of the controls and it was suggested that there was an increase in gonadotropin secretion.

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THE EFFECT OF GROWTH HORMONE ON THE ANEMIA INDUCED BY HYPOPHYSECTOMY IN ADULT FEMALE RATS[1]

It has been well established that hypophysectomy induces an anemia. In an attempt to prevent this anemia, hypophysectomized rats have been injected with thyroxine (Meyer, Thewlis, and Rusch, 1940; Vollmer, Gordon, and Charipper, 1942; Crafts, 1946a). The erythrocyte count was maintained at normal values, but the hemoglobin decreased to sub-normal levels. Androgen therapy (Vollmer and Gordon, 1941; Vollmer, Gordon, and Charipper, 1942; Crafts, 1946b) produced similar results. Crafts (1949) treated hypophysectomized rats with daily injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate. In addition, the rats were given a high protein diet ad libitum. This therapy actually prevented all aspects of the anemia which follows hypophysectomy.

The present experiment was undertaken to determine whether growth hormone would prevent anemia in hypophysectomized rats.

Received for publication March 19, 1953.

[1] This work was done with the aid of a contract between the University of Cincinnati and the Office of Naval Research, Washington, D. C.

The methods used have been described previously (Crafts, 1949). The Long-Evans strain of rats has been used in this study. Rats were fed *Purina* Chow ad libitum which was supplemented once a week with lettuce.

The results obtained are shown in Table 1. Data are presented for normal untreated rats, hypophysectomized rats with no treatment, and for hypophysectomized rats treated with growth hormone. The intact untreated rats exhibited no significant changes in the blood picture during the 50 days of observation. The body weight of these rats

TABLE 1. THE EFFECTS OF GROWTH HORMONE THERAPY ON THE ANEMIA THAT IS INDUCED BY HYPOPHYSECTOMY

Day = Day of treatment; BW = Body weight in gm.; TRC = erythrocyte count in millions per cu. mm.; Hemat = Hematocrit reading in per cent; HB = Hemoglobin in gms. per 100 cc; MCV = Mean corpuscular volume in cu. microns; MCH = Mean corpuscular hemoglobin in micromicrograms; MCHC = Mean corpuscular hemoglobin concentration in per cent; ± = Standard deviation.

Day	BW	TRC	Hemat	HB	MCV	MCH	MCHC
			Normal Controls—11 rats				
0	175	7.91	42.9	13.8	54.2	17.4	32.0
	±27.0	±0.60	±2.0	±0.6	±2.5	±0.9	±0.5
10	191	7.71	41.7	13.2	54.2	17.2	31.9
	±30.0	±0.62	±2.1	±0.8	±1.9	±0.6	±1.0
20	199	7.34	40.6	12.6	55.6	17.1	30.9
	±32.0	±0.74	±2.9	±1.1	±2.3	±0.7	±1.1
30	202	7.30	41.3	12.9	56.6	17.7	31.3
	±27.8	±0.60	±2.9	±1.0	±3.7	±1.0	±1.0
40	203	7.15	41.3	12.6	58.3	17.7	30.5
	±29.8	±0.92	±2.8	±1.4	±5.5	±1.3	±2.4
50	205	7.37	42.9	13.3	58.7	18.1	31.0
	±29.2	±0.82	±2.3	±0.9	±6.7	±1.4	±1.2
			Hypophysectomy—20 rats				
0	193	7.86	42.9	13.6	54.6	17.3	31.7
	±12.8	±0.36	±1.7	±0.7	±2.0	±0.7	±0.7
10	168	8.20	44.7	14.6	54.5	17.8	32.6
	±9.1	±0.52	±3.4	±1.1	±2.0	±0.8	±0.9
20	162	7.59	40.9	13.0	52.5	17.1	32.6
	±8.7	±0.66	±1.6	±0.9	±2.4	±0.8	±0.7
30	151	6.84	34.8	11.5	51.0	16.9	33.1
	±9.0	±0.74	±3.0	±1.0	±2.2	±0.8	±1.1
40	148	5.93	30.6	10.2	51.8	17.2	33.2
	±6.5	±0.60	±2.5	±0.9	±0.9	±0.9	±1.0
50	140	5.72	28.0	9.5	49.0	16.6	33.9
	±9.3	±0.44	±1.8	±0.6	±2.9	±0.9	±1.3
	Hypophysectomy followed by growth hormone therapy as follows: days 1–9, 0.1 mg.; days 10–19, 0.2 mg.; days 20–29, 0.3 mg.; days 30–39, 0.4 mg.; days 40–49, 0.8 mg.—7 rats						
0	180	7.96	42.9	13.9	54.0	17.5	32.5
	±6.7	±0.51	±1.2	±0.6	±3.0	±1.1	±0.9
10	166	7.84	42.9	13.5	54.6	17.2	31.5
	±10.1	±0.41	±1.9	±0.9	±1.7	±0.6	±1.0
20	175	5.59	31.4	9.5	57.7	17.0	29.8
	±9.3	±1.50	±5.3	±2.4	±7.5	±1.3	±3.4
30	178	5.68	32.1	9.8	58.1	17.4	30.4
	±10.8	±1.00	±2.2	±1.4	±9.2	±1.2	±2.7
40	183	5.35	29.2	9.0	55.2	16.8	30.6
	±11.1	±0.87	±2.9	±1.4	±5.2	±1.1	±2.3
50	192	5.73	31.2	9.6	54.6	16.8	30.9
	±15.0	±0.62	±2.1	±0.7	±4.2	±1.1	±1.2

increased from an average of 175 gm. at the beginning of the experiment to 205 gm. 50 days later.

Hypophysectomy induced the usual anemia. In these untreated rats, the erythrocyte count decreased from 7.86 ± 0.36 million cells per cu. mm. before hypophysectomy to 5.72 ± 0.44 million cells on the 50th day after hypophysectomy; the hematocrit, from $42.9 \pm 1.7\%$ to $28.0 \pm 1.8\%$; and the hemoglobin, from 13.6 ± 0.7 gm. per 100 cc of blood to 9.5 ± 0.6 gm. These hypophysectomized untreated rats also exhibited a significant decrease in mean corpuscular volume (Table 1). This decrease in erythrocyte volume did not induce a significant decrease in mean corpuscular hemoglobin because the concentration of hemoglobin in each erythrocyte increased slightly. Hypophysectomy induced a decrease in body weight from an average of 193 gm. at the time of hypophysectomy to 140 gm. 50 days later.

Growth hormone,[2] injected subcutaneously and in gradually increasing dosage, as indicated in Table 1, had no effect on the erythrocyte count, hematocrit, or the hemoglobin of hypophysectomized animals. After 50 days of treatment, the erythrocyte count was 5.73 ± 0.62 million cells per cu. mm., the hematocrit reading was $31.2 \pm 2.1\%$, and the hemoglobin level was 9.6 gm. per 100 cc of blood. The growth hormone treatment did prevent the decrease in mean corpuscular volume found in the untreated hypophysectomized rats. The body weight of these treated hypophysectomized rats increased from an average of 180 gm. at the time of hypophysectomy to an average of 192 gm. after 50 days of growth hormone treatment. The effects of growth hormone on the erythrocyte count, hematocrit reading, and hemoglobin level of hypophysectomized rats in this study are similar to those reported by Meyer, Stewart, Thewlis and Rusch (1937) and by Van Dyke, Simpson, Garcia and Evans (1952).

Growth hormone is known to promote protein anabolism. Since it did not prevent anemia in the hypophysectomized animals, doubt is cast on the importance of faulty protein metabolism as a cause for the development of an anemia in the hypophysectomized rat. There is a possibility that the beneficial effects obtained by treating hypophysectomized rats with thyroxine, testosterone propionate, and high protein diet (Crafts, 1949) may have been due to the thyroxine and androgen rather than the high protein diet. This point is being investigated.

In summary, hypophysectomized rats were treated with gradually increasing doses of growth hormone in an attempt to prevent the anemia which is induced by removal of this gland. Although this hormone prevented the decrease in body weight and the decrease in mean corpuscular volume which occurred in the untreated hypophysectomized rats, it had no effect on the erythrocyte count, hematocrit, or on the total amount of hemoglobin.

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UNSATURATED COMPOUNDS IN HUMAN URINES[1]

In a previous publication we reported the presence of an unidentified unsaturated compound m.p. 144–144.5° (Miller and Dorfman, 1950). The clinical description of this patient (Wisconsin General Hospital No. 127483), who presented symptoms of virilism, has been described (Schiller *et al.* 1945). This compound has now been identified as Δ^{16}-androstene-3α-ol. This paper also reports the isolation of Δ^4-androstene-3, 17-dione from the urine of a woman with adrenal cortical carcinoma.

EXPERIMENTAL

Isolation of Δ^4-androstene-3, 17-dione

Urine was collected for a week pre-operatively and combined with urine collected post-operatively on 4 days (17-ketosteroid values ranging from 20–672 mg. per day) over a 5 month period. Following hydrolysis with 50 cc. concentrated sulfuric acid per liter of urine for 10 minutes at reflux temperature, the urine was twice extracted by shaking for ½ hour with 200 cc. butyl ether per liter of urine. From the butyl ether residue the neutral ketonic, digitonin non-precipitable fraction was prepared by the use of Girard's reagent T, and digitonin. This material (weight 1 gram, containing approximately 900 mg. 17-ketosteroids) was chromatographed and twice rechromatographed on aluminum oxide (Brockmann). Elution with benzene yielded approximately 3 milligrams of crystals, m.p. 163.5°–172°. When these crystals were mixed with Δ^4-androstene-3, 17-dione, m.p. 168.5°–172°, the melting point was 167°–172°. The compound showed an absorption maximum at 242 mμ, and gave a positive Samuels reaction (Koenig, Melzer, Szego and Samuels, 1941). The infra-red spectrum of the isolated compound was identical to that of Δ^4-androstene-3, 17-dione.

Isolation of Δ^{16}-androstene-3α-ol

The compound (12 mg.) was originally isolated from the non-ketonic fraction of a urine sample (76.1l.) from a virilized woman (Schiller *et al.*, 1945). The urine extract was prepared by extracting the fresh urine with benzene without acid hydrolysis. The free compound melted at 144°–145.5°C and when mixed with an authentic sample (Dr.

Received for publication April 23, 1953.

[1] Supported in part by Research Grant G-3247, Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service and Contracts DA-49-007-MD-184 and DA-49-007-MD-310, Medical Research and Development Board, Office of the Surgeon General, Department of the Army, and by contract AT (30-1)-918. U. S. Atomic Energy Commission.

H. L. Mason) (m.p. 144.5°–145°C) the melting point was 145°C. The acetate melted at 132°–132.5°C and when mixed with an authentic sample (m.p. 129.5°–130.5°C) the melting point was 130°–132°C. The infra-red spectrum of the free compound was identical to that of the authentic sample.

DISCUSSION

The isolation of Δ^{16}-androstene-3α-ol from human urine confirms the finding of Mason (1950). This compound is the 14th steroid to be isolated from the urine of this particular patient with (probable) adrenal hyperplasia (Miller and Dorfman, 1950).

Δ^4-androstene-3,17-dione has been isolated from the urine of a man with adrenal hyperplasia (Lieberman *et al.*, 1948), from the adrenocortical extracts (von Euw and Reichstein, 1941), from the blood perfused through a human testis (Savard, *et al.*, 1952), and from the spermatic vein blood of the dog (West *et al.*, 1952).

SUMMARY

Δ^4-androstene 3,17-dione has been isolated from urine of a woman with adrenal cortical carcinoma. Δ^{16}-androstene-3α-ol has been isolated from the urine of a woman with probable adrenal hyperplasia.

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FAILURE OF CORTISONE TO INFLUENCE THYROID FUNCTION

If cortisone influences thyroid function, the characterization of this influence would be quite important in the understanding of problems of interendocrine relationships and endocrine control. Many reports recently have indicated that cortisone depresses some tests of thyroid function, and an extensive—and conflicting—literature has appeared

regarding possible sites of action (see references). There is, however, a more basic controversy, in which the occurrence of any influence by cortisone on the thyroid is questioned (see review by Albert, 1952). Because of these conflicts, it seems desirable to report some of our observations.

Albino rats[1] and healthy young adult dogs, both on a diet containing 0.5% iodized salt, were studied. The indices of thyroid function employed were plasma protein-bound iodine (PBI) concentrations, thyroid weights, and thyroid acinar cell height indices (CHI). The CHI is the mean height of cells in 100 consecutive acini.

EXPERIMENTAL

1. Four dogs were treated with 100 mg. cortisone acetate (Merck) intramuscularly daily for 45, 60, 195, and 199 days. Weekly determinations of plasma PBI concentration were done during treatment. There were no significant changes in plasma PBI concentration during cortisone administration.

2. The relative thyroid weight and CHI of dogs treated with cortisone did not differ from these measurements in an untreated control series. Comparatively, hypophysectomy was associated with atrophy and thyrotropin administration with hypertrophy of the thyroid. The results are shown in Table 1.

3. Rats 45 days old were given 3 mg. of cortisone acetate subcutaneously daily for

TABLE 1. RELATIVE THYROID WEIGHTS AND CHI OF DOGS

Series	Daily dose	No. of dogs	Mean thyroid wt., mg/kg B. W.	CHI, micra
Controls	——	6	89.7 ± 10.2	5.3 ± 0.07
Cortisone 30 days	100 mg.	5	87.7 ± 12.5	5.7 ± 0.51
Cortisone 45–199 days	100 mg.	5	85.0 ± 6.9	5.6 ± 0.72
Hypophysectomy (>1 month)	—	5	55.4 ± 4.4*	3.4 ± 0.16†
Thyrotropin 12 days	5 mg. b.d.	1	119.8	9.5

* $p < 0.02$.
† $p < 0.01$.

30 days. Treated and control animals were pair-caged and -fed. Thyroid weights, CHI, and PBI of these animals are presented in Table 2. Cortisone did not significantly alter relative thyroid weight, CHI, or PBI in the female rats. There was an *increased* plasma PBI in the male rats, but no significant alteration in the thyroid weight or CHI was observed. All treated animals showed the usual adrenal and thymic atrophy.

4. Female rats 45 days old were given a solution of 1% thiourea as their only available drinking water for 30 days. Half of these were given 3 mg. of cortisone acetate daily subcutaneously. The results are shown in Table 3. The concurrent administration of cortisone did not interfere with the goitrogenic action of thiourea.

SUMMARY

Cortisone, given for prolonged periods to dogs and rats, did not alter thyroid weight

Received for publication April 30, 1953.

[1] Holtzman-Rolfsmeyer.

TABLE 2. THYROID WEIGHTS, CHI, AND PBI OF RATS

Group	No. of rats	Final body wt. g.	Absolute thyroid wt., mg.	Relative thyroid wt., mg/ 100 g. BW	Mean CHI micra.	Mean PBI, μg%
Female controls	4	219±4.1	13.4±0.98	5.9±0.32	6.1±0.86	3.6±0.25
Female cortisone	3	188±4.5*	11.2±0.91	5.9±0.33	6.8±0.32	3.8±0.21
Male controls	3	336±9.2	18.0±0.57	5.4±0.27	7.7±0.62	3.7±0.10
Male cortisone	3	273±3.9*	16.6±1.13	6.1±0.64	9.6±1.35	4.5±0.08*

* $p < 0.01$.

TABLE 3. CONCURRENT ADMINISTRATION OF CORTISONE AND THIOUREA TO RATS

Group	No. of rats	Final body wt., g.	Absolute thyroid wt., mg.	Relative thyroid wt., mg./ 100 g. BW	Mean CHI, micra.	Mean PBI, μg.%
Untreated controls	3	178±6.6	14.5±2.4	8.1±1.1	5.2±0.32	3.6±0.25
Thiourea	5	135±3.2*	45.1±2.7*	33.4±2.0*	11.7±4.2*	2.6±0.46‡
Thoiurea +cortisone	5	90±7.9†	45.1±1.2*	50.2±2.2†	12.2±0.47*	2.6±0.12*

* $p < 0.01$ (comparison with controls).
† $p < 0.01$ (comparison with controls and with thiourea group).
‡ $p < 0.10$.

or histology and did not consistently alter plasma protein-bound iodine concentrations. Cortisone did not interfere with the goitrogenic action of thiourea in rats.

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[2] Recipient of grants-in-aid from the United States Public Health Service and the Commonwealth Fund.

FAILURE OF METHYLANDROSTENEDIOL TO PREVENT COMPENSATORY ATROPHY OF THE ADRENAL IN THE RHESUS MONKEY[1]

The recent observations by Winter, Hollings and Stebbins (1953) and Gaunt, Tuthill, Antonchak and Leathem (1953) that methylandrostenediol (MAD) prevents the cortisone induced adrenal atrophy in the rat has engendered much interest in the possible clinical applications of MAD with regard to the adrenal inhibiting effects of cortisone in primates.

The present communication relates an attempt to repeat the above observations in the rhesus monkey.

MATERIALS AND METHODS

Six immature female monkeys were utilized in this study. Three of these received 50 mg.of cortisone acetate per day for 33 days and three, in addition to the same cortisone regimen, were given 50 mg. of MAD[2] per day for the same period. Both hormones were injected intramuscularly at different sites. Prior to and during the injection period body weights were recorded at weekly intervals and the blood levels of glucose, non-protein nitrogen, inorganic phosphorous, sodium, potassium and chloride determined (Greep, Knobil, Hofmann and Jones, 1952). Weekly hematocrit determinations were also made. Glucose tolerance tests were carried out before the initiation of treatment and during the last week of hormone administration (Knobil, Hofmann and Greep, 1953).

The day following the last injection, the animals were sacrificed by an overdose of pentobarbital sodium and the various organs listed in Table 1 were weighed. The adrenal glands were fixed in 10% neutral formalin and Zenker-formol and subsequently prepared for staining with eosin and hematoxylin, sudan B and the detection of birefringence and autofluorescence.

RESULTS AND DISCUSSION

The previously reported effects of cortisone (Knobil, Hofmann and Greep, 1953) on body weight and organic as well as electrolyte metabolism in the rhesus monkey were not modified by the simultaneous administration of MAD. The atrophy of the adrenal glands observed following the injection of cortisone was not inhibited by MAD, Table 1. The results also indicate that ovarian, pituitary, kidney and thyroid weights were not significantly different in the two groups of animals. The weight of the uteri, on the other hand, were significantly greater in those monkeys receiving MAD in addition to cortisone. This uterine enlargement was associated with a striking reddening of the sex skin and of the facial epithelium. These color changes were not accompanied by an increase in the turgescence of these structures. A marked hypertrophy of the clitoris was also observed.

No significant differences could be detected between the adrenals of the two groups with regard to general histological configuration and the distribution of sudanophilic, birefringent and autofluorescent materials. The inclusion bodies characteristic of adrenal cortical cells in the rhesus monkey (Knobil, Rosa and Greep, 1953) were absent in cortisone and cortisone-MAD treated animals.

The failure of MAD to prevent the compensatory atrophy of the adrenal in response

[1] Aided in part by grants from the American Cancer Society and The United States Public Health Service.

[2] We are indebted to Dr. Robert Gaunt of Ciba Pharmaceutical Products, Inc. for generous supplies of methylandrostenediol.

TABLE 1. EFFECTS OF CORTISONE AND CORTISONE + MAD ON INTACT, IMMATURE FEMALE MONKEYS

Treatment	Monkey no.	Pretreatment body weight gm.	Body weight at autopsy gm.	Left adrenal mg.	Right adrenal mg.	Kidneys gm.	Uterus mg.	Ovaries mg.	Thyroid mg.	Pituitary mg.
50 mg/day cortisone for 33 days	E1	3815	3965	161	136	26	488	119	464	46
50 mg/day cortisone for 33 days	E2	3700	3650	188	152	21	1049	194	567	39
50 mg/day cortisone for 33 days	E3	3820	3650	232	186	20	1050	163	464	50
	Mean	3776	3755	194	158	22	862	159	498	45
50 mg. cortisone +50 mg. MAD /day for for 33 days	M1	3650	3755	181	161	23	1754	166	780	48
50 mg. cortisone +50 mg. MAD /day for 33 days	M2	3560	3600	154	180	20	1595	181	402	42
50 mg. cortisone +50 mg. MAD /day for 33 days	M3	3540	3600	184	143	19	1776	72	339	34
	Mean	3583	3652	173	161	21	1708	140	507	41

to cortisone in these experiments may be attributed to a non-optimal ratio of cortisone to MAD, although such a ratio (1:1) was found to be effective in the rat (Gaunt, 1953). The possibility remains, however, that the adrenal-maintaining activity of MAD and related compounds as observed in the rat is not demonstrable in all species.

CONCLUSIONS

Methylandrostenediol at the dose level employed, did not modify the cortisone-induced atrophy of the adrenal cortex in the rhesus monkey.

(See next page for Acknowledgement and References)

Acknowledgement

The technical assistance of Mrs. L. W. Parker and Miss A. Morse is gratefully acknowledged.

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